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FUSARIUM WILT OF CARNATION IN KENYA AND ITS INTERACTION WITH VESICULAR-ARBUSCULAR MYCORRHIZAE

by

VIRGINIA NJUGUINI MWAI

©

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE
OF

MASTER OF SCIENCE

IN

PLANT PATHOLOGY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

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"The irreconcilable conflict between science and religion is that science is never fixed-it is open and progressive: religion, on the other hand, is fixed on absolutes and dogmas"

Anon.

".....they shall mount up with wings like eagles"

Is. 40:31

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....

Dr. J. P. Tewari (Supervisor)

....

(Dr. N. R. Knowles)

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(Dr. B. Ooraikul)

Date: 12|12|92

To the women who have been such a positive influence in my life;
Shirley E. Kabachia, Nelius N. Kabachia,
and Teresa W. Mwai, my Mom.

Abstract

Vascular wilt of carnation in Kenya was found to be caused by *Fusarium oxysporum f. sp. dianthi*. Five isolates of this pathogen whose cultural characteristics differed on half-strength PDA were isolated from wilted carnation stems and arbitrarily designated B, E, F, G, and H. Isolates B and E differed significantly from isolates F and G with respect the maximum disease severity (K_{max}). Isolate H was found to be intermediate (alpha = 0.01). *Fusarium avenaceum* was also isolated from the wilted carnations and caused stem and bud rot of carnation upon re-inoculation.

The carnation cultivar Lolita developed a functional symbiosis with *Glomus intraradices* and *G. mosseae*, but not with *G. dimorphicum*. Inoculation with VAM fungi enhanced the severity of Fusarium wilt in Lavender Lace, but depressed disease severity in Portrait and Scania. Phosphorous content did not differ between the VAM inoculated plants and the uninoculated plants (alpha = 0.01). The dry weights of VAM inoculated plants differed significantly from those of uninoculated plants for Lavender Lace, but not for Portrait and Scania (alpha = 0.01).

The spores of *G. mosseae, G. fasciculatum, G. intraradices* and *G. dimorphicum* were found to retain their viability and infectivity with 13 months of cryopreservation in liquid nitrogen. Viability staining with 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was found to successfully differentiate viable from non-viable spores of *G. mosseae* and *G. fasciculatum*. This stain, however, failed to differentiate between viable and non-viable spores of *G. intraradices* and *G. dimorphicum*.

The carnation growing areas of Kenya were found to have wide variety of VAM fungi species. Fifteen different species of VAM fungi spanning the genera *Glomus, Acaulospora, Entrophospora,* and *Gigaspora* were isolated and identified from the soil samples collected from these regions.

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Chapter I

Literature Review

A. Introduction

Carnation (*Dianthus caryophyllus* L.) culture in Kenya is mainly in the rift valley region 35-100 km North West of Nairobi city and 70-110 km south of the Equator. The region is 1700-3000 m above sea level. Miniatures are grown in open fields without protection at an altitude of 1800 m. Standards are grown at higher altitudes of 2700 m and above, either under polyethylene structures (large scale production) or in the open fields (small scale production). The carnations produced feed a growing local market, and are also exported to Europe, USA, Japan, and the Middle East, providing much needed foreign exchange. The carnation industry employs more than 4000 people at the primary production level (Cox, 1987; Ministry of Agriculture, Nairobi, Kenya Per. comm.). In the last five years, vascular wilt of carnation caused up to 100% crop loss on some farms in the Kiambu area of Kenya, leading to the abandonment of carnation culture by many farmers (Ministry of Agriculture Reports, Kiambu, Kenya).

B. Vascular Wilt of Carnation

Vascular wilt diseases cause major losses and have been a constant threat to carnation culture worldwide. Phialophora wilt, caused by *Phialophora cinerescens* (Wollenw.) Van Beyma, was the most important disease in carnation culture up to 1970. This disease was eventually largely eliminated, probably due to the use of drenches with systemic fungicides (Garibaldi, 1978), the increased availability and accessibility of pathogen-free plant material (Baker, 1980), as well as improved cultural methods such as the maintainance of high temperatures in greenhouses (Sparnaaij and Demmink, 1975).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *dianthi* (Prill. & Del.) Snyder & Hansen (*F. o. dianthi*) only became important in carnation culture after 1970 (Baayen, 1988). Within the last two decades, it has become the most important disease of carnation, threatening the sustainability of carnation culture world-wide. It often causes very severe losses, and once introduced into a carnation growing area, subsequent elimination of the pathogen is extremely difficult. Steam sterilization and chemical fumigation of contaminated soil are not usually satisfactory means of control for the disease, and neither is use of systemic fungicides (Garibaldi, 1978; Gamboa, 1986; Garibaldi and Gullimo, 1987; Evans, 1978; Margraf et al., 1986).

C. Causative Agents

Phialophora cinerescens is a hyphomycete with short, dark, simple or branched phialides, that are either cylindrical or inflated. The conidia are subhyaline to dark, one celled, globose to ovoid, and they extrude from phialides in moist heads (Barnett and Hunter, 1987). The fungus was first described by Wollenweber (1929) (as quoted by Baayen, 1988) as *Verticillium cinerescens* Wr., but was later included by Van Beyma (1940) in the genus *Phialophora* as, *P.*

cinerescens. Previously, the disease caused by this fungus has been referred to as Verticillium wilt (Brown, 1938 and Guba, 1947). It is a slow growing fungus requiring a relatively low temperature of 11°C for optimal growth and sporulation *in vitro* (Sparnaaij and Demmik, 1975).

The causative agent for Fusarium wilt was originally described as *F. dianthi* Prill. and Del. It was recognised by Wollenweber and Reinking (1935, as quoted by Baayen and Gams, 1988.) who placed it in the section Elegans, subsection *oxysporum*, together with *F. oxysporum* Schlecht : Fr., *F. vasinfectum* Atk. and *F. redolens* Wollenw. The section was reduced to a single species by Snyder and Hansen (1940), *F. oxysporum*, of which the carnation pathogen was considered a *forma*; *F. oxysporum* Schlecht. f. sp. *dianthi* (Prill. & Del.). Gerlach (1961 as quoted by Baayen and Gams,1988) restored *F. redolens* as a separate species based on macroconidial morphology. The discovery of *F. redolens* strains causing vascular wilt in carnation therefore led Gerlach and Pag (1961 as quoted by Baayen and Gams,1988) to conclude that there exists two morphologically different Elegans Fusaria, *F. redolens* f. sp. *dianthi* and *F. o dianthi*, that cause wilt disease of carnation, although the diseases caused by both are indistinguishable (Gerlach and Pag, 1961; Hantschke, 1961). This distinction between the two pathogens was questioned by Baayen and Gams (1988) who found that on the basis of morphology, the two pathogens form one variable complex. Distinction between *F. oxysporum* f. sp. *dianthi* and *F. redolens* f. sp. *dianthi* could not be justified either at the specific or the varietal level (Baayen and Gams, 1988).

D. Fusarium Systematics

A fundamental problem inherent in *Fusarium* identification is that members of the genus vary widely in their morphological and non-morphological characterisitics, including virulence. These criteria are used in taxonomic systems. Several taxa within the genus are recognized by the International Code of Botanical Nomenclature (based on morphology) and include section, species and variety (Windels, 1991). There are also some subdivisions within *Fusarium* species based on physiology (*formae speciales* and races) or genetics (vegetative compatibility groups) that are not recognized by the code according to Article 4.3 (Greuter, 1988).

Each section of *Fusarium* contains species that share common morphological characterisics (Windels, 1991). When Snyder and Hansen (1940) revised the Elegans section, the concept of *forma speciales* was applied to recognize pathogenic strains that were morphologically indistinguishable from saprophytic strains of the same species, but differed in their ability to parasitize specific hosts with the belief that *formae speciales* were specific to one host. In reality, host specificity occurs in some, but not all *formae speciales* (Armstrong and Armstrong, 1981). Further subdivisions of *formae speciales* into races are often made based on virulence to a particular set of differential host cultivars.

E. Pathogenesis of Fusarium Wilt in Carnation

The pathogen is able to penetrate the epidermal cells of young carnation roots by means of appressoria-like structures (Baayen, 1987). Penetration is then followed by intracellular growth of hyphae within epidermal and cortical cells, and into the vascular tissue. Baayen (1988) noted however that the fungus prefered a direct access to the root stele by entering through wounds.

The fungus may also enter the host by direct access through exposed vessels at the base of the stem. The presence of such exposed vessels is a consequence of the vegetative propagation (cuttings) used in carnation culture. Sparnaaij et al. (1989) showed that the exposed vessels at the cut surface of a cutting may remain open for prolonged periods. Colonization remains restricted to the xylem until the host is severely diseased, and it proceeds mainly vertically (hence unilateral symptoms). The colonization is accomplished by mycelial growth rather than by passive dispersal of conidia and is therefore continous (Pennypacker and Nelson, 1972; Baayen, 1988).

F. Control Of Fusarium Wilt of Carnation

Despite routine soil disinfection with fumigants prior to each crop, and the use of cuttings obtained from Fusarium-free mother plants produced from meristem cultures, vascular wilt incited by *F. o. dianthi* continues to cause economic losses in all carnation growing areas of the world. Soil reinfestation with *F. o. dianthi* after disinfection may originate from infected plant residues present below the depth affected by disinfection, introduction of infected cuttings and/or through cultural practices (Garibaldi and Gullino, 1987). Introduction of *F. o. dianthi* by wind, which has for a long time been considered unimportant, may be very important, especially in carnations grown on artificial substrates (Tramier, 1986 as quoted by Garibaldi and Gullino, 1987).

1. Steam Disinfection

Eradication of the pathogen through steam disinfection and chemical fumigation of the soil is frequently incomplete. Steam disinfection, although offering the best results and being relatively environment friendly is very expensive and creates a biological vacuum favorable to quick establishment of other pathogens such as *F. roseum* Lk. emend. Snyd. & Hans. and *Rhizoctonia solani* Kühn (Garibaldi and Gullino, 1987).

2. Soil Fumigation

Soil fumigants: methan-sodium, dazomet and methyl-isothiocynate, strongly reduce *Fusarium* spp. inoculum, but it has been shown that recolonization by *F. o. dianthi* after fumigation is rapid. This is a result of the negative influence fumigation has on soil suppressiveness, by reducing the total amount of non-pathogenic Fusaria (Cugudda and Garibaldi, 1986).

Soil fumigation with methyl bromide controls carnation vascular wilt (Besemer and McCaine, 1978), but large amounts of water (80-100 L/m^2) are needed to leach out the toxic bromide residues before planting (Vigodsky-Haas and Klien, 1976). Methyl bromide is highly efficient due to its ability to reach the pathogen at a depth of 40-50 cm. Solarization of soil is effective against Fusarium wilt (Garibaldi and Gullimo, 1987) and can be a viable option especially in the tropics.

3. Fungicidal Control

Fungicidal control (e.g. using benzimidazoles) has been shown to give variable, and at times negative results (Evans, 1978; Gamboa, 1986). Phthalimides (captafol, captan) when applied as soil drenches or as dust showed higher activity for partially resistant cultivars grown in heavily infested soils than for susceptible cultivars (Gullino and Garibaldi, 1984). Carbendazum drenches applied soon after planting are more effective than drenches delayed until after the onset of symptoms. The decrease in disease with carbendazum drenches only prevents loss of flower production at low *F. o. dianthi* inoculum levels (Ebben, 1977). Although effective against fungal vascular wilt, soil treatments have been reported to fail, especially when systemic fungicides are used due to acquired resistance by the pathogen (Leski, 1977, Tramier and Bettachini, 1974; Gullino et al.,1986).

4. Cultural Control

Ammonium nitrogen decreases wilt severity when compared with nitrate nitrogen. Nitrite, rather than ammonia is responsible for the decline effect of ammonia generating compounds on populations of *F. o. dianthi* in soil (Loffler et al., 1986). Soil pH of 7.5 or higher definitely decreases disease severity, while amending soil with peat or sulphur decreases soil pH and increases disease severity (Garibaldi and Gallino, 1987). Calcium deficiency increases disease severity during any stage of plant development (Blanc et al., 1983). Pera and Calvet (1989) reported that the use of composted olive pumice or a commercial composted pure bark instead of sphagnum peat in container media delayed appearance of wilt in carnations.

5. Resistance

Severe epidemics of Fusarium wilt have led to large reductions in acreage devoted to the carnation industry in areas of southern Europe (Italy and France), stimulating research on the development of carnation varieties resistant to the wilt pathogen (Garibaldi and Gullino, 1987). However, the presence of several races of *F. o dianthi* and its ability to differentiate into new pathotypes which can overcome Fusarium wilt resistance complicates the search for resistant cultivars (Garibaldi, 1983). Due to the partial nature of resistance to *F. o. dianthi*, screening for resistant cultivars is very time consuming and delicate, especially in comparison with the urgent need for new varieties. (Sparnaaij, 1983). Maintainance of quality and productivity are proviso in commercial carnation breeding, and selections not adhering to the stringent standards of plant and flower quality are generally eliminated. Even if a breeder succeeds in developing resistant lines, floricultural characteristics must be equal to, or exceed the quality of the existing cultivars, which is difficult even for breeders not selecting for resistance (Garibaldi and Gullino, 1987).

Resistance of carnations to Fusarium wilt may operate at various stages of the infection process. Inhibition or retardation of fungal growth may occur when the fungus attempts to penetrate the epidermis, cortex, or endodermis of roots (extravascular resistance), but also in the xylem of roots and stem (vascular resistance) (Baayen et al., 1991) In the xylem, resistance generally involves confinement of the invading fungus at the infection site by means of physical and chemical barriers such as the formation of vessel-occluding gums, and accumulation of fungistatic compounds such as phenolics and phytoalexins (Niemann and Baayen, 1988; Baayen and Niemann, 1989). The localization in xylem is similar in roots and stems (Baayen, 1988; Baayen et al., 1989). Tolerance does not seem to play any part in the resistance mechanism of carnations against *F. o. dianthi* (Baaayen, 1988). Regeneration of xylem may create an alternative pathway for water transport to compensate for that lost by occlusion or maceration due to disease (Harling et al., 1985).

6. Biological Control

The difficulties encountered in eradicating Fusarium wilt pathogen discussed above make control with biological agents an attractive and useful addition to the available disease control measures. Soils that are suppressive to *F. o. dianthi* have been reported (Schroth and Hancox, 1981; Scher and Baker, 1980, McCain et al., 1980; Tramier et al., 1983). Disease suppressiveness has a microbiological origin that is destroyed by steaming. Certain bacteria and fungi indigenous to these soils have been shown to be responsible for suppression of Fusarium wilt pathogens (Garibaldi, 1984). All suppressive soils transferred suppressiveness to disease conducive soils when added in small amounts (2-10% w/w) (Scher and Baker, 1980; McCain et al., 1980), and successive cultivation of susceptible plants increased soil suppressiveness. The most efficient of these microorganisms were identified as *Pseudomonas* and *Alcaligenes* in the U.S.A and nonpathogenic Fusaria in France and Italy. Suppressiveness induced by *Pseudomonas* spp. and *Alcaligenes* spp. was attributed to their superior iron siderophore stability compared to that of pathogenic *Fusarium* spp., giving them an edge in competition for iron (Peer et al., 1990; Yuen and Schroth, 1983). *Fusarium oxysporum* conidia need iron for germ tube elongation and competition for iron at the rhizoplane between suppressive microorganisms producing siderophores and pathogenic *Fusarium* spp., induces suppressiveness. As a consequence, management systems that selectively reduce the amount of iron available to pathogens for starting the infection can induce soil suppressiveness (Scher and Baker, 1982). Tramier et al. (1983) attributed soil suppressiveness to *F. o. dianthi* to antagonistic non-pathogenic Fusaria. Postma and Rattink (1992) found that non-pathogenic Fusaria, both wild-type and benomyl resistant mutants, suppressed

Fusarium wilt in a susceptible cultivar of carnation by 80%. Suppression of wilt in carnation by non-pathogenic isolates of *Fusarium* is also reported by Tramier et al. (1987).

Pre-treatment of plants with nonpathogenic microorganisms can reduce the severity of the disease (Biles and Martyn, 1989; Hillocks, 1986). Treatment of plants with abiotic stimuli can also reduce disease symptoms (Anchisi et al., 1985; Bovio et al., 1987). This phenomenon of biotic and abiotic pretreatment to affect resistance is called induced resistance. Both induction and protective effects are non-specific, and the mechanism is indirect and mediated by the plant (Kroon et al., 1991). Systemic induced resistance in controlling Fusarium wilt in carnation has been described involving a *Pseudomonas* sp. isolate and a nonpathogenic *Fusarium* isolate. (Peer et al., 1991). However, with nonpathogenic Fusaria, degree of disease suppression has been found to give inconsistent results (Postma and Rattink, 1991).

Chitinolytic bacteria isolated from carnation rhizospheres and applied by dipping roots of carnation in cell suspension during transplanting, are also successful biocontrol agents against Fusarium wilt (Garibaldi and Gullino, 1987). Significant reduction in the number of diseased plants under field conditions, by applying cell suspensions of *Arthrobacter* sp. and *Serratia liquefaciens*, has been reported (Sneh, 1981; Sneh et al., 1985). To date use of chitinolytic bacteria has not been applicable in carnation culture at an industrial level and soil suppressiveness can only be maintained where present, but cannot be easily introduced (Garibaldi and Gullino, 1987).

G. Vesicular-arbuscular-mycorrhizae Fungi as Biocontrol Agents of Plant Disease

1. Host Range

The term "mycorrhiza" literally means "fungus root" (Powell and Bagyaraj, 1984). It was first coined by Frank (1885 as quoted by Powell and Bagyaraj, 1984) to describe plant-fungus associations. Mycorrhizae are formed by a diverse group of fungi comprising Ascomycetes Basidiomycetes and Zygomycetes (Harley and Smith, 1983). Zygomycetous endomycorrhizae are the most abundant and widespread, and are found in the roots of most plant families so far examined, although they may be rare or absent in families such as Cruciferae, Chenopodiaceae, Cyperaceae, and Caryophyllaceae (Tester et al., 1987). The term vesicular-arbuscular mycorrhizal (VAM) has been used as a synonym for zygomycetous endomycorrhizae because of the fungal structures they produce in root cortex (vesicles and arbuscules), although some of the fungal symbionts do not produce intraradical vesicles (Morton, 1988). The fungal symbionts are ubiquitous, soil-inhabiting biotrophes that have potential as biological control agents of soil-borne diseases. Vesicular-arbuscular mycorrhizae (VAM) fungi are ubiquitous and form obligate symbiotic relationships with most agricultural crops (Caron, 1989). Although fungi are not host specific, they exhibit certain host preferences (Daniels Hetrick, 1984). Most plant species normally have mycorrhizas, but some plant taxa (nonmycorrhizal) do not usually form generally recognizable mycorrhizas (Tester et al., 1987). In considering VAM fungi, most workers accept a plant as mycorrhizal if arbuscules are present, indicating the establishment of a functional symbiosis on the grounds that arbuscules are believed to be the main site of nutrient transfer (Hirrel et al. 1978). However, host plants may also modify the structure and behavior of a mycorrhiza which develops on it and different hosts may form different structures from the same species of VAM fungus (Boyetchko and Tewari, 1990). Some 'nonmycorrhizal' plants can have infection by VAM fungi induced in their roots when grown in the presence of a mycorrhizal host (Hirrel et al., 1978).

2. Interaction of VAM Fungi With Carnation

Carnations belong to the order Caryophyllales which is described as 'nonmycorrhizal' (Tester et al.,1987). However, not all Caryophyllales are nonmycorrhizal. For example the Cactaceae is clearly a mycorrhizal family (Reeves et al., 1979; Rose, 1981). In the order Caryophyllales, which has a total number of 9,700 species, only 2% of these have been examined for mycorrhizal susceptibility. Of the 2%, 13% were susceptible to zygomatous (VAM) fungi and 62% were found to be nonmycorrhizal (Trappe, 1987).

3. Mechanisms of Resistance to VAM Fungal Infection

The presence of fungitoxic compounds in root cortical tissue and root exudates may reduce the susceptibility of plants to VAM infection (Bevege and Bowen, 1975). Tommercup (1984) found that *Brassica napus* L. produces soluble or volatile compounds that reduce the rate of germination of spores of mycorrhizal fungi and the rate of hyphal growth outside roots, but do not lower the maximum percent germination or hyphal extension. Reduction of root exudation has been suggested as a reason for low mycorrhizal infection in plants (Graham et al., 1981). Many workers (Bevege and Bowen, 1975; Hirrel et al., 1978; Ocampo et al., 1980) have noted that hyphae can grow profusely over the surface of roots of a weakly mycorrhizal plant, but can never penetrate the root. This implies that barriers to infection may have more to do with mechanical or chemical characteristics of root epidermis or cortex than with any phenomena occuring outside the root such as exudation. A study done by Bencard and Piche (1990) on carrot (Umbelliferae) and sugar beet (Chenopodiaceae) indicates that the nonmycorrhizal roots of sugar beet lack factors that promote mycorrhizal activity, rather than producing inhibitory factors. In addition, Mercy et al. (1990) revealed that mycorrhizal colonization is not only host dependent, but is also a heritable trait in a study involving cowpea (*Vigna unguiculata* L. Walp.).

4. Biocontrol of Diseases Caused by Soil-borne Fungi

Vesicular-arbuscular mycorrhizal fungi have been reported to protect plant roots from certain root infecting fungi (Caron, 1989) and are therefore potential biological control agents for soil-borne plant pathogens. *Glomus fasciculatum* (Thaxter sensu Gerd.) Gerd. & Trappe reduced *Pythium ultimum* Trow propagule density in the rhizosphere of mycorrhizal greenhouse grown poinsettia (Kaye et al.,

1984) Leading to reduced disease severity. Hwang et al., (1992) found that inoculation of alfalfa (*Medicago sativa* L.) with VAM fungi reduced the incidence of Fusarium and Verticillium wilts. They also found that VAM fungi reduced propagule numbers of the wilt causing agents, and concluded that VAM fungi possibly produce antimicrobial compounds. Caron et al. (1986a, 1986b, 1986c) were able to reduce severity of crown and root rot of tomato caused by *Fusarium oxysporum* Schlecht f. sp. *radicis-lycopersici* Jarvis & Shoemaker using *Glomus intraradices* Schenck & Smith. They also found that inoculation of plants with *G. intraradices* reduced the number of propagules of the pathogen. Well watered *Asparagus officinialis* L. inoculated with root rot causing *F. oxysporum* were less diseased and sustained lower rhizosphere populations of the pathogen when they were preinoculated with *Glomus fasciculatum* (Wacker et al., 1990). However, VAM has also been shown to increase disease severity. Davis et al. (1979) noted that the incidence of Verticillium wilt of cotton was enhanced by inoculation with *G. fasciculatum*. Modjo (1983) found that *G. macrocarpum* did not affect severity of Burley tobacco stunt disease. Vesicular-arbuscular mycorrhizae have also been found to be ineffective in altering pathogen-induced plant disease severity. Davis (1980) did not find any significant reduction of root rot in citrus with VAM fungi inoculation.

5. Mechanisms of Suppression of Pathogens by VAM Fungi

Disease resistance in the host plants could be due to morphological alterations caused by mycorrhizal infection. Thickening of the cell walls through lignification and production of other polysaccharides in mycorrhizal plants may prevent the penetration of pathogens. In a study involving Fusarium wilt of tomato caused by *F. oxysporum* f.sp. *lycopersici*, Denhe and Schonbeck (1979b) associated improved tolerance to Fusarium wilt of mycorrhizal tomato plants with increased lignin synthesis, and its ecrustation in the stele region. This was as a result of increased phenol synthesis by the plants. Krishna and Bagyaraj (1983) have observed an inhibition *in vitro* by *Sclerotium rolfsii* Curzi by concentrations of O-D phenols that were comparable to those found in mycorrhizal peanut plant roots. Morandi et al. (1984) found increased concentrations of three isoflavonoids in mycorrhizal soybean (*Glycine max* (L) Merr.) compared to non-mycorrhizal, and concluded this was an indication that mycorrhizal plants respond more quickly to pathogen attack than do the non-mycorrhizal plants. Baltruschat and Schönbeck (1975) associated the increased resistance of tobacco to *Thielaviopsis basicola* (B. & Br.) McCormick with increased arginine content of the mycorrhizal roots.

Alteration of disease severity in mycorrhizal plants could also be due to physiological and biochemical changes in the host induced by the mycorrhizal fungi. It is well established that VAM fungi improve absorption of nutrients, particularly phosphorus in mycorrhizal plants (Abbot and Robson, 1984). Improved phosphorus nutrition has been associated with reduction in disease severity (Davis et al., 1979; Graham and Menge, 1982; Kaye et al., 1984; Krishna and Bagyaraj, 1983). Conversely, Davis et al. (1979) indicated an increase in Verticillium wilt of cotton with improved P nutrition, while Bååth and Hayman (1983) and Caron et al. (1986b) indicated that the protection effect was not due to improved P nutrition. Graham and Menge (1982) explained the decline of take-all disease of wheat by a diminution of root exudates resulting from improved P nutrition of mycorrhizal plants. On the other hand, Caron et al. (1986b) showed that P concentrations in the plant had no effect on Fusarium root rot of tomato, while the presence of *G. intraradices* resulted in protection of the root system.

H. Systematics of VAM Fungi

A typological perspective of morphology and germination characteristics of anamorphs has been used to classify VAM fungi in the division Eumycota, and class Zygomycetes, partly due to their chitinaceous cell walls. A study done on lipid and fatty acid profiles of four species suggested that VAM fungi have closer links with protoctistan fungi than Zygomycetes (Jabaji-Hare, 1988). This suggestion was discredited by Morton (1990), who is of the opinion that the lipid component is a primitive zygosporic character that can not be used to define more highly evolved monophyletic groups of Zygomycetes. The ultimate criterion for placement of a fungus in the Zygomycetes is the formation of zygospores, although these are not commonly formed by many VAM fungi in a natural setting. Members of the genus *Endogone* and *Gigaspora decipiens* have been found to produce zygospores (Gerdemann and Trappe, 1974; Tommerup and Sivasithamparam 1990). The latter finding clearly places at least one VAM fungus firmly in the zygomycetes.

Endomycorrhizae are further placed in the order Endogonales and family Endogonaceae (Trappe and Schenck,1982; Morton,1988). Six genera (*Acaulospora* Gerdemann & Trappe emend. Berch, *Entrophospora* Ames & Schneider, *Gigaspora* Gerdemann & Trappe, *Glomus* Tulasne & Tulasne, *Sclerocystis* Berkeley & Broome, and *Scutellispora* Walker & Sanders) whose members form arbuscular mutualistic symbioses with many terrestrial families (Trappe, 1987) were included, as well as the type genus Endogone Link:Fr. The genus *Endogone* is the only one classified according to characteristics of the teleomorphic stage (sporocarpic zygospores) and its members are saprobic (Gerdemann and Trappe, 1974) or form putative ectomycorrhizal associations (Chu-Chou and Grace, 1979).

A burgeoning number of new species descriptions in recent years have etablished a reasonable database of organismal diversity to assess stability and distribution of germination and morphological characters (Morton, 1988; 1990; Pirozynski and Dalpe, 1989; Almeida et al., 1990). However, absence of distinctive interspecific differences in mycorrhizal anatomy at the light microscope level has discouraged more detailed analyses for classification purposes (Morton, 1990a). Morton (1988) reviewed morphological and germination characters of reprodutive and vegetative structures as identification criteria for VAM fungi. The extent to which these characters established a pattern of evolutionary change among known taxa has been analysed by Morton (1990a) using 57 species from six genera of VAM fungi. He describes members of the genus *Endogone* as a polyphyletic group related to some arbuscular species in *Glomus* and *Sclerocystis* only by convergence. He also hypothesized evolution of two main branches from a common arbuscular ancestor. One branch consisted of the genera *Gigaspora* and *Scutellospora*, as defined by extraradical auxiliary cells and spores formed within a thin unit wall on a sporogenous cell. The other branch consisted of *Glomus*, *Sclerocystis* *Acaulospora* and *Enthrospora* as defined by the presence of intraradical vesicles in mycorrhizal roots.

The mentioned data, along with ontogenic evidence, has been used to revise the classification of VAM fungi to reflect more natural groupings. The fossil record has temporally linked the geological appearance of *Glomus* and *Sclerocystis* to each other (Stubblefield and Taylor, 1988). Differences between these two genera have become less distinct with the description of presumed dimorphic species: *G. ambisporum* Schenck & Smith, *G. heterosporum* Smith & schenck, and *G. dimorphicum* Boyetchko & Tewari which show chlamydospores organized in a pattern similar to that found in *Sclerocystis* leading to speculation that the two genera are synonymous (Walker, 1987). On this basis, the two genera were placed in an additional family, Glomaceae, characterized by "chlamydospores" borne singly, in aggregates, or in compact sporocarps on one or more cylindrical to flared subtending hyphae (Pirozynski and Dalpe 1989) .

In a revised classification of VAM fungi, Morton and Benny (1990) introduced a new order, Glomales, to include all soil borne fungi that form arbuscules in obligate mutualistic associations with terrestrial plants. They proposed a sub-order Glominae, comprising the type family Glomaceae and Acaulosporaceae fam. nov., the latter comprising *Acaulospora* spp. and *Enterophrospora* spp.. Acaulosporaceae are distinguished by "chlamydospores" formed laterally from or within a hypha terminating in a sporiferous saccule. Gigasporineae subord. nov. was to include Gigasporaceae fam. nov. is also proposed. The genera in this family, *Gigaspora* and *Scutellospora* produce "azygospores" borne terminally on a sporogenous cell. Thus the taxa in Gigasporineae produce extraradical auxiliary cells and no intraradical vesicles, whereas taxa in the Glominae form intraradicle vesicles. They also amended the Endogonales order to contain one family Endogonaceae, with a single genus, *Endogone*.

Almeida and Schenck (1990) studied spore developement in *Sclerocystis* and *Glomus*. Based on their findings, the genus *Sclerocystis* was maintained with one species, *S coremioides* Berk & Broome. *Sclerocystis coccogena* (Pat.) von Höhn and *S. dussii* (Pat.) von Hohn are considered synonyms of *S coremioides*. Five other *Sclerocystis* spp. are moved to the genus *Glomus*, to become *G. clavisporum* (Trappe) Almeida & Schenck, *G. rubiforme* (Gerdemann & Trappe) Almeida & Schenck, *G. sinuosum* (Gerdemann & Bakshi) Almeida & schenck, *G. liquidambaris* (Wu & Chen) Almeida & Schenck, and *G. taiwanese* (Wu & Chen) Almeida & Schenck. The remaining five described species. of *Sclerocystis* were considered synonyms of the aforesaid *Glomus* species.

I. Identification of VAM Fungi

This involves recognition of the fungal spore or group of spores. Descriptions are based on spore characteristics (Schenck and Perèz, 1990; Morton, 1988). Physical characteristics of spores can be visualized universally with a good light microscope. This enables comparison of spore characteristics using preserved specimens, species descriptions, illustrations and keys, and therefore gives spore morphology primacy in classification and identification of VAM fungi (Morton, 1988).

Criteria used in identification include spore organization either within root or sporocarp; morphology, size, shape, and texture of sporocarps; and spore size, shape, color and morphology (Morton, 1988). The spore wall morphology more than any other characteristic defines the fungus. Spore wall types are identified strictly by their appearance in intact or broken spores under a light microscope (Schenck and Pérez, 1990). Walker (1983) first defined the evanescent, unit, laminated and membranous wall types. The expanding wall was described by Berch and Koske (1986), the amorphous wall by Morton (1986), and Walker (1986) described the coriaceous wall. Murographs are used to represent wall type, number from spore surface, and group as proposed by Walker (1983).

Reaction of the spore wall with Melzers reagent (Melzer, 1924 as quoted by Morton, 1988) aids in identifyig VAM fungi. A positive reaction results in a color change in the fungal structures. A blue or amyloid reaction has been attributed to the presence of straight chained A-1,4 glucosidic-linked polysaccharides (McCracken and

Dodd, 1971), while a red or dextrinoid reaction is attributed to presence of high concentrations of quaternary ammonium compounds (QACs) which form a peroxide complex with potassium iodide (Blackwell et al., 1985). The true amyloid reaction has not been observed in walls of VAM fungi, but a gradation of color between blue and red, as well as the dextrinoid reaction have been reported (Morton, 1986b; Schenck et al., 1984; Sieverding and Toro, 1987; Trappe and Janos, 1982).

Both chemical and mechanical effects need to be considered when observations are being made (Morton, 1988). Mountants have been shown to affect the integrity of both spore color (Koske and Walker) and spore wall structure (Morton, 1986b; Morton and Koske, 1988). The observed wall groups will depend on the amount of pressure applied to break the spores. The amount of pressure needed will depend on the mountant viscosity (Morton, 1988). The morphology of sporogenous hypha, sporiferous saccule, and the occlusion of spore contents are also criteria used in VAM fungi identification. The morphology of the fungus in root has been considered as a possible identification criterion (Abbot, 1982), but was later shown to be host dependent (Boyetchko and Tewari, 1990).

Some non-morphological characteristics used recently in VAM fungi taxonomy include biochemical techniques (Hepper et al., 1988), serological identification of spores (Wright et al., 1987), gas chromatography of cell contents (Weijman and Meuzelaar, 1979), and DNA homology assessment (Miller, 1987).

Currently, at least 148 species of VAM fungi are described in literature. The absence of any means to confirm or establish identity of these fungi prevents consolidation of biological and ecological characteristics at the species level. Identification is therefore mostly based on broad, inadequate description (Abbot and Robson, 1991; Morton, 1988). This problem is exacerbated by the general inavailability of living cultures of specific VAM fungi that have been described. Few experimental cultures have been maintained or preserved in personal or institutional herbaria (Morton, 1988). A case in point is the "*Glomus fasiculatum*" complex which has been cited as one species in studies performed worldwide, giving an impression of a ubiquitous organism with considerable genetic and physiological plasticity based on broad descriptions by Gerdemann and Trappe (1974) (Morton, 1988). A more restrictive description of the fungus has led to the segregation of many isolates previously identified as *G. fasciculatum* into different taxa (Walker and Koske, 1987). Systematics of VAM fungi is therefore still poorly developed with most work still in a descriptive phase (Morton, 1990) and identification is therefore not sinecure. Even where clear descriptions have been prepared, it can still be difficult to identify certain species if isolated spores are too few, or spores are present at different stages of developement, or if the fungus forms spores too small to be easily recovered from soil (Abbot and Robson, 1991, 1982).

J. Distribution of VAM Fungi

These taxonomic difficulties have made the determination of individual species distribution difficult (Abbot and Robson, 1982). Vesicular-arbuscular mycorrhizal fungi are ubiquitous in soils

(Hetrick et al., 1984). They occur mostly in the top 20 cm of the soil profile (Redhead, 1977) and there is a general exponential decline of both infection and spore number with increase in depth (Zajicek et al., 1986). A few instances where these fungi may be absent include eroded soils (Habte, 1989), fumigated soils or soils disturbed by mining (Jasper et al., 1987).

The fungi are dispersed primarily by wind (Tommerup and Carter, 1982; Warner et al., 1987) and soil transportation. Because of this ability to disperse and their extensive host range, VAM fungi are known to be widely distributed in environments ranging from tropical rain forest to arctic tundra (Janos, 1980). An analysis of some common species of VAM fungi suggests that they show little habitat specificity (Mosse and Bowen, 1968, Walker et al., 1982). Ecotypic differentiation has been reported for some species (Hepper et al., 1988; Adelman and Morton, 1986). A study done by Stahl et al. (1990) supports the hypothesis that populations of *Glomus mosseae* (Nicol. & Gerd.) Gerdemann & Trappe from dissimilar environments are genetically different races or ecotypes suggesting a possibility of significant genetic and physiological diversity within this morphologically defined taxon. Haas and Krikun (1985) reported differences in growth responses by different strains of VAM fungi, but provided no evidence to support the veracity of species/strain identification.

The distribution of VAM fungi is known to vary with climatic and edaphic environment, as well as with land use (Abbot and Robson, 1991). Being obligate symbionts, VAM fungi distribution is greatly affected by vegetation (Kucey and Paul, 1983; Thompson, 1987). Crop species have been known to exert a selective effect on the abundance of different species of VAM fungi in a mixed indegenous population (Schenck and Kinlock, 1980). The presence of so called non-hosts seem to be better for VAM fungi than no plant at all, as VAM fungi have been observed to colonize their root surfaces (Matarè and Hattingh, 1978) thereby procuring an ecological niche in which to survive in the absence of host root. This raises the possibility of some saprophytic growth in the rhizosphere soil by VAM fungi.

K. VAM Fungi in the Tropics

Mycorrhizas in the tropics have recieved much less attention than in temperate zones and most information on the physiology of mycorrhizas comes from temperate-zone studies (Bowen, 1980). Surveys of mycorrhizal hosts and fungi in native tropical vegetation are sporadic and a bulk of experimental investigation deals primarily with effects of VAM on crop growth (Bowen, 1980). Thus discussion of the eco-physiology of VAM in the tropics seems to require a temparate/tropical comparison and extrapolation of data from temparate-zone fungi (Janos, 1987). This can be potentially misleading because of probable adaptation of VAM fungi to edaphic conditions (Lambert et el., 1980).

Unusually few VAM fungal spores are found in tropical forest soils which may be attributed to loss of VAM fungi after forest clearing. This leads to a lower grade forest soil. However, most tropical tree species that have been examined, form VAM associations (Janos, 1983), which may be indispensable for seedling survival and growth. Högberg (1982) demonstrated a dominance of endomycorrhizal species in indigenous Tanzanian trees and shrubs studied. In Miombo woodlands, a major ecosystem in East and South-central Africa, ectomycorrhizal trees predominate by volume, while endomycorrhizal species dominate by numbers (Högberg, 1982).

Oxisols and ultisols account for two thirds of world tropical soils by area. These are clays with good physical characteristics, well drained, but chemically deficient They are acid, have high potential aluminium toxicity, low cation exchange capacities indicative of high leaching potential, low available potassium, calcium, magnesium, sulfur, zinc, and micronutrients, and are known as "phosphate fixing" soils due to their high capacity to convert soluble phosphates to insoluble forms (Sanchez, 1976).

The hyphae of VAM fungi extend into the soil well beyond the rhizosphere, effectively extending the zone of nutrient absorption, especially for poorly mobile elements such as phosphorus, copper and zinc (Mukerji et al., 1991), and thus enhancing their uptake by plants (Ames et al, 1983). As regulatory agents of plant nutrition, VAM fungi have more use in the tropics than in temperate zones (Diem, et al., 1981).

L. Production and Application of VAM Fungi

Exploitation of VAM fungi for agricultural use is dependent on either producing commercially viable inoculum on plant hosts, or manipulating agricultural systems to develop and exploit indigenous

VAM populations (Simpson and Daft, 1990), as the fungi are considered obligate symbionts that cannot be grown in pure culture (Hepper, 1984). The difficulties involved in producing, storing, and applying VAM fungi inoculum in field crop systems preclude any easy usage of the fungus, even when growth benefits are likely (Hayman, 1987). Of the various techniques devised for introducing VAM fungi inoculum into the field grown crop, use of preinoculated transplants is one of the simplest, when appropiate (Hayman, 1987). Adhesives such as methyl-cellulose inoculated with VAM fungal spores that have been concentrated by wet-sieving can be used to suspend germinated seed and this is then applied as a slurry in seed furrows. This has been successful in field inoculation of red clover (Hayman et al., 1981), but is impractical for small seeded crops (Hayman, 1984). Increasing density of VAM fungi *in situ* by growing a heavily mycorrhizal crop in a rotation aystem is time and labour consuming. Topsoil transffered into an area from another area as VAM inoculum, also introduces to the area, new microflora which may contain plant pathogens.

To date, the large quantities of VAM fungi inoculum needed for field inoculation is usually raised in pot cultures, which have a high propensity to become contaminated either by other VAM fungi (Hall, 1977), or other contaminants, that may be plant pathogenic (Hayman, 1987). Inoculum has also been successfully produced in aeroponic culture resulting in well colonized root systems and proliferation of spores (Hung and Sylvia, 1988). If plants could be inoculated without soil, then contamination of VAM fungi cultures by root pathogens, hyperparasites, and other VAM fungi could be eliminated (Hung et al., 1991). Aseptic, viable VAM fungi spores have been successfully produced (Tommerup and Kidby, 1980) and several hydrogels can be used as sticking agents for direct inoculation of VAM fungi spores onto host plant roots (Hung et al.,1991; Johnson and Hummel, 1985) thereby avoiding contamination. On the other hand, deliberate contamination of VAM inoculum with compatible micro-organisms that are beneficial to plants could be a useful innovation (Hayman, 1987). Since the stock cultures are raised in sterilized soils, selected micro-organisms could be introduced and established before invasion and competition from aerial contaminants becomes intense. Bacteria that dissolve insoluble phosphates, produce growth-promoting compounds, or are antagonistic to specific plant pathogens might be introduced into the rhizosphere this way (Hayman, 1987). One should take into consideration the fact that some micro-organisms may inhibit germination of specific VAM fungi (Tommerup, 1985), and others stimulate it (Daniels et al., 1980).

M. Storage and preservation of VAM fungi

In viability tests performed on *Gigaspora margarita* Becker & Hall, Sward et al. (1978) found that germination percentage and viability of spores decreased markedly with storage in all the different conditions of storage tested. They concluded that the best way to store VAM spores is to leave them in their native soil and store whole sample in cold rooms. Methods of storing VAM fungal inoculum that allow for higher inoculum concentration and therefore require less storage space merit further investigation (Hayman, 1987). Studies on the viability of VAM fungi inocula after physical and chemical stresses provides insight into the ability of these fungi to survive laboratory operations, and some understanding of their behaviour in their normal ecological niche (Tommerup and Kidby, 1980). Daft and Spencer (1987) investigated infectivity of VAM fungal spores, hyphal fragments, mycorrhizal, and root segments after storage under various environmental conditions. They found that the three inocula types differ in their inoculum potential and survival during storage. Whereas VAM fungal spores exhibit dry heat resistance (Tommerup and Kidby, 1979), they do not survive moist heat at 60 C (Tommerup and Kidby, 1980). These two researchers also reported that VAM fungi spores can be decontaminated using oxidizing agents, antibiotics and ultrasonic radiation without necessarily rendering the spores unviable. They therefore concluded that it is possible to develop a protocol for producing large numbers of uncontaminated spores. Such spores can then be used for long-term preservation of VAM fungi.

N. Objectives of the thesis

This study was undertaken with four objectives in mind.

1. To study the etiology of vascular wilt of carnation in Kenya
2. To identify some of the VAM fungi indigenous in the carnation growing area of Kenya.
3. To study the interaction of *F. o. dianthi* and VAM fungi on carnation.

4. To explore the possibility of cryopreservation of VAM fungi spores in liquid nitrogen.

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Chapter II

Etiology of Vascular Wilt of Carnation in Kenya

A. Introduction

Phialophora wilt, caused by *Phialaphora cinerescens* and Fusarium wilt caused by *Fusarium oxysporum* f. sp. *dianthi* are the two most important vascular wilt diseases of carnation. The symptomatology of the two diseases can be quite distinctive. The symptoms of Fusarium wilt are often unilateral. Externally, the afflicted foliage becomes chlorotic and finally necrotic. Internally, the vascular system has a light brown discolouration. With Phialophora wilt, the whole plant gradually wilts, leaves fade and become straw coloured, and the vascular system has a 'chocolate brown' discolouration (Baker, 1980). Symptomatology however, can be subjective, and is a function of the environment as well as the host and pathogen genotypes (Garibaldi, 1975; 1978; Demmink et al. 1989). Etiology becomes the most reliable diagnostic feature distinguishing between the two diseases.

Phialophora cinerescens is a hyphomycete with short, dark, simple or branched phialides, that are either cylindrical or inflated. The conidia are subhyaline to dark, one celled, globose to ovoid, and they extrude from phialides in moist heads (Barnett and Hunter, 1987). Spore morphology is the major identifying characteristic of Fusaria. All *Fusarium* species have one taxonomic feature in common: potential to produce falcate, (1-)3-5(-9) septate, pedicellate macroconidia, with phialidic conidiogenesis, and tapered or curved apical cells (Booth, 1984; Gams and Nirenberg, 1989; and Pascoe, 1990). A distinction is usually made between macroconidia, which are often sporodochial, and microconidia, which are borne on aerial mycelium. Microconidia are ellipsoid, ovoid, subglobose, pyriform, clavate or allantoid, 0-(1) septate with a rounded truncate base. They are phialidic, and accumulate in small slimy false heads or chains (Pascoe, 1990). Chlamydospores, when present may occur singly, in pairs, chains or clumps, and may be formed in the macroconidia or the hyphae. They may also aggregate into microsclerotia (Nelson et al., 1983). Pascoe (1990) defined a third type of conidium, termed as the mesoconidium. Mesoconidia are straight or curved, fusoid, lanceolate, clavate or falcate, 0-5(-7) septate with a simple, narrowly truncate apedicellate basal cell. They do not accumulate in slimy heads or sporodochia, but remain dry, often giving aerial mycelium a powdery appearance.

There are eight races of *F. o.* f. sp. *dianthi* recognized presently (Garibaldi, 1983). Race 2 occurs wordwide (Baayen et al., 1988). Race identification based on host-pathogen interactions is complicated by qualitative and quantitative variation (Garibaldi, 1983). Factors such as type and level of resistance and susceptibility, isolate virulence and aggressiveness, inoculum density and mechanism of pathogenesis, all affect host-pathogen interactions (Garibaldi and Gullino, 1987; Tramier et al., 1987 and Baayen et al., 1988). Pathogenicity tests do not indicate genetic relatedness among isolates of a given physiologic race or *forma speciales*. In absence of a known sexual stage in *F. oxysporum,* heterokaryosis has been used as an alternative means of determining genetic relatedness among

Fusarium strains (Pullaha, 1985). Isolates that are able to anastomose and form stable heterokaryons constitute a vegetative compatibility group (VCG). Pullaha (1985) further showed that isolates in different *formae speciales* of *F. oxysporum* were in distinct vegetative compatibility groups. *Fusarium oxysporum* f. sp. *dianthi* race 2 is a uniform population composed of a single VCG and constitutes a distinct genetic population within the *F. oxysporum* complex (Katan and Katan, 1989).

The purposes of the present study were to: (a) determine the causative agents of carnation vascular wilt in Kenya; (b) determine the pathogenicity of the different isolates on one susceptible carnation cultivar, under greenhouse conditions.

B. Materials and Methods

1. Sampling of Diseased Carnation Plants

A carnation wilt disease survey was carried out in Kiambu and Limuru carnation growing areas to: 1. establish the presence and extent of disease. 2. observe the cultural practices of Kenyan carnation culture.

Carnation stem sections were collected from field grown carnations that exhibited typical vascular wilt symptoms (wilted shoots, chlorotic to straw colored foliage, stunting, and a striking internal brown discoloration of the vascular tissues). Sampling was arbitrary. The collection was done from the central part of Kenya, where carnation vascular wilt is prevalent.

2. Isolation of the Pathogens

The stem pieces were surface sterilized in a 3.5% sodium hypochlorite solution for 10 min., plated onto Potato Dextrose Agar (PDA) (Difco) slants, and incubated at 22 C and a 12 hr. photoperiod for 14 days. The slants were then sealed with parafilm and stored at 4 C until shipped to Canada (for about one month).

Small squares of agar containing fungus were cut out from the outer edges of the fungal slants and transfered onto one-half strength PDA plates. The inoculated plates were incubated for 16 days at 25 C under a 16 hr. photoperiod. From the 16-day-old cultures, single spore cultures were made as described by Nelson et al. (1983), and grown on one-half strength PDA for 18 days under conditions as described above. From the same slants, squash mounts were prepared and the fungal morphology studied under a compound light microscope.

3. Identification of Pathogens

Cultural characteristics such as abundance and color of aerial mycelium, presence and colour of sporodochia, presence of sclerotia and color of colony reverse, were used to group the 18-day-old cultures into eight different isolates, arbitrarily designated A, B, C, D, E, F, G, and H. Slants of each isolate were sent to National Identification Service, Agriculture Canada, Ottawa where they were identified following species concepts of Nelson, Toussoun, and

Marasas (Nelson et al., 1983). The conidial morphology of each of the eight isolate types was also observed under a light microscope.

4. Pathogenesis of Isolates

Rooted carnation cuttings of the Fusarium wilt susceptible cv. Portrait were obtained from Yoder Carnations. The eight isolates described above were inoculated onto a mixture of oat and wheat seed (2:1: V:V) and incubated for 14 days at room temperature. The infected seed was then homogenized using a waring blender and passed through a 0.5 mm pore sieve. The sieved inoculum was stored at 4 C until needed. Rooted carnation cuttings were planted singly in 10 cm pots in a steam sterilized sand:loam:peat:moss:vermiculite mix (3:1:1:1:1). The eight isolates were used to inoculate ten pots each. Each pot was inoculated with 7.2×10^7 colony forming units (CFU) of the inoculum (determined in a previous experiment). The inoculum was placed directly below and in contact with the roots of the cuttings. Each pot comprised an experimental unit, each of the eight isolates comprised a treatment, and there were ten replicates for every treatment. As a control, ten pots were not inoculated. A completely randomized design was used. The pots were placed in a growth cabinet with temperatures maintained at 20 C night, 24 C day, and 70-85% RH with regular watering. Full-strength Hoaglands solution was used to fertilize the plants at 100 ml $pot^{-1}week^{-1}$. Four shoots were maintained per plant through regular thinning.

Disease symptom development was monitored and recorded every seven days for a period of 90 days after inoculation. The plants were scored for disease using a five-point index as follows: (Modification of one used by Demmink et al. 1989)

0 = No apparent symptom or necrosis.

1 = Slight wilt and/or chlorosis

2 = Moderate wilt; when about one half of the plant was wilted

3 = Severe wilt; when a major portion of the plant was wilted but not fully dead

4 = The whole plant was wilted and fully necrotic.

(Fig. II- 0 - II-5)

The scores were then converted to percentage disease incidence using the following formula.

$$DS = \{ [(0) \times 0] + [(1) \times 1] + [(2) \times 2] + [(3) \times 3] + [(4) \times 4] \} \times 100/4n$$

where:

DS = Disease severity

n = Number of plants per treatment (in this case 10).

() = Number of plants in indicated index point

At the end of the experiment, the pathogen was reisolated from the diseased carnation stems, plated out, squash mounts made, and the conidial morphology examined under a light microscope.

5. Data Analysis

The data was fitted to the logistic model for comparison of rates of disease progression. The proportions of the disease severity values were transformed into logits to linearlize them using the following formula from Campbell and Madden (1990)

$$Yl = \ln [Y/(1-Y)] = \ln [Y_o/(1-Y_o)] + r_l t$$

Linear regression was then used to estimate the rate parameter ($r_l t$) and the Y intercept (Y_o). An empirical approach was used whereby the model used was determined by the data set. Disease indices at the maximum disease severity levels for the five *F. o dianthi* isolates were put through analysis of variance procedures on SAS (SAS Institute Inc., Cary, NC) to determine whether the K_{max} (maximum disease severity) were significantly different from one another.

C. Results

1. Disease Survey

Carnation vascular wilt was observed in the two main carnation growing areas, Kiambu and Naivasha. Disease severities on the farms ranged between 10-100% and in some cases led to the abandonment of carnation culture. In the Naivasha area, production is mainly large-scale. One carnation growing company (Sulmac Co. Ltd, Naivasha) produces carnations on 137.4 ha while small scale growers (with farms <0.5 ha) produce carnations on a total area of 2.6 ha. The Sulmac company purchases disease-free material once a year and bulk up a supply for production plantings. They also use fungicidal drenches as well as soil fumigation to keep vascular wilt at a minimum level. The smallscale farmers do not purchase disease-free material nor do they practice chemical control of vascular wilt. They do however use crop rotation.

Kiambu area had mainly small-scale producers with a few midscale producers (0.5-5 ha). Chemical control of the disease in this area was negligible. Propagation material was obtained either from farmers own previous crop or bought from street vendors. The farms in this region had vascular wilt intensities of up to 100%.

2. Identification of Pathogen Isolates

Isolates A-H (Figs. II-6 - II-10) were identified as follows according to the species concept of Nelson, Toussoun and Marasas (1983).

ISOLATE	DAOM#	NAME
A	213389	*Fusarium equseti* (Corda) Sacc. sensu Gordon
B	n/a	*Fusarium oxysporum* Schlecht.
C	23390	*Fusarium avenaceum* (Fr.) Sacc.
D	23390	*Fusarium avenaceum* (Fr.) Sacc.
E	n/a	*Fusarium oxysporum* Schlecht.
F	213391	*Fusarium oxysporum* Schlecht.
G	213391	*Fusarium oxysporum* Schlecht.
H	213391	*Fusarium oxysporum* Schlecht.

(DAOM#- accession number at the National Identification Service, Agriculture Canada, Ottawa).

3. Gross Morphology and Light Microscope Studies of Isolates

I. *F. equiseti* (Fig. II-11)

On half-strength PDA, the aerial mycelium was white. Orange sporodochia with a slimy consistency were produced. The colony reverse varied in color from tan to brown. Both branched and non-branched monophialides were produced. Abundant microconidia (0-1 septate) borne on heads were observed. These were mainly oval in shape. A few fusoid to falcate mesoconidia with a simple truncate basal cell were observed. Macroconidia were abundant. They were prominently septate, thick-walled, and sickle-shaped with a very distinctive curvature. The dorsal curvature was more abrupt than the ventral one. The apical cell was extended and the basal cell distinctively foot shaped. Chlamydospores were abundant, with prominent, thick, and roughened walls. There were both terminal as well as intercalary chlamydospores which formed in clumps and chains. The chlamydospores formed only in the mycelium, and not in the macroconidia.

II. *F. avenaceum* (Fig. II-12)

On half-strength PDA, the aerial mycelium was dense and white with a pink to red brown tint. Orange sporodochia were present, and the colony reverse color varied from tan to carmine red. Both branched and non-branched monophialides were produced.

Very few microconidia and mesoconidia were seen. Macroconidia were abundant, long (2-6 septate), slender, thin walled, and had an elongated apical cell. The basal cell was pedicellate. Chlamydospores were absent.

III. *F oysporum* f. sp. dianthi

a. Colony morphology (on half-strength PDA)

Isolate B

White aerial mycelium was produced. The colony reverse had a light blue purple coloration. No sclerotia were observed.

Isolate E

The aerial mycelium was at first white, but rapidly acquired a deep purple coloration. The colony reverse was a deep purple. There were no sclerotia observed. The culture was not found to be contaminated as had been indicated by National Identification Service, Agriculture Canada, Ottawa

Isolate F

The aerial mycelium was white and eventually became tinged with a pink purple color. Abundant submerged blue-black sclerotia were observed. The sclerotia consisted of compact masses of chlamydospores.

Isolate G

This isolate was morphologically similar to Isolate F except that it was not observed to form sclerotia.

Isolate H

The aerial mycelium was white with prominent sporodochia. The colony reverse was a light salmon pink, however, when sclerotia were present, the colony reverse appeared blue-black.

b. Conidia and conidiophore morphology (Fig. II-13)

In all the isolates, the microconidia were abundant, oval to kidney shaped. The macroconidia, also abundant, were only slightly sickle shaped. They had an attenuated apical cell and a foot shaped basal cell. Mesoconidia with appedicellate foot cells were also observed, but were scarce. Branched monophialides were observed. Chlamydospores formed profusely. both in the hyphae, and the macroconidia.

4. Pathogenicity tests

F. equiseti

This isolate did not cause any apparent symptoms on the carnation cultivar Portrait. However, it was reisolated from the basal part of the stems of the inoculated plants.

F. avenaceum

This species did not cause vascular wilt, but was pathogenic to carnation cv. Portrait. At the inoculum level of 7.2×10^7 CFU, all inoculated plants became straw colored and died within seven days. At 3.6×10^6 CFU of inoculum, the inoculated plants became progressively chlorotic starting from the lower leaves. Inoculated plants were all visibly stunted at the end of the experiment (about 1/3 height of the control) and they did not produce any side shoots (Fig. II-14 and II-15). At the end of the experiment, the inoculated plants showed some vascular tissue discoloration (brown), mainly towards the stem base. *Fusarium avenaceum* was reisolated from the inoculated plants at the end of the experiment.

F. oxysporum

All the *F. oxysporum* isolates caused vascular wilt to the inoculated plants. The first wilt symptom of isolate B was consistently a crook neck. This was followed by wilt with little or no chlorosis (up to 40% of affected leaf area). The chlorosis was unilateral and more often than not involved only the midrib. The leaf margins had a reddish-brown discoloration.

Isolate E produced similar symptoms except that it produced a brighter chlorosis than all the other strains.

Plants inoculated with H, G, and F portrayed a higher degree of chlorosis (up to 80% of affected leaf area). All infected plants had a brown vascular discoloration. All strains could be re-isolated from the respective inoculated plants.

5. Disease progression

The disease progression curves had slopes ranging from 0.05 - 0.11 (Table II-1). The number of days before onset of visible disease symptoms differed among isolates, ranging from 31 - 52, and the K_{max} (maximum disease severity value) ranged from 20% - 92.5% (Fig. II-16). The K_{max} values were found to be significantly different by the (Student-Newmans-Keuhl) SNK test at alpha = 0.01 level of significance (Table II-2). On the basis of K_{max} values two isolates, B and E, were not significantly different from one another. Isolate F was not significantly different from isolate G. The latter two, however, differed significantly from the former two isolates. Isolate H was intermediate between B and E on one hand, and F and G on the other. On the basis of the disease progression curve slope, Isolate B had the steepest slope at 0.106. Isolates E with a slope of 0.074 was similar to Isolate H with a slope of 0.066. and Isolates F with a slope 0.05 was similar to Isolate G with a 0.049 slope (Table II-1).

D. Discussion

Vascular wilt of carnation in Kenya was found to be caused by *F oxysporum* f. sp. *dianthi* and not *Phialophora cinerescens*. This is not surprising because the carnation growing region in Kenya has an average temperature of 24 C (Nairobi Meteorological Station, personal communication), and *P. cinerescens* requires a relatively low temperature of 11 C for optimum growth and sporulation (Sparnaaij and Demmink, 1976). The disease survey and subsquent sampling of diseased carnation plants carried out was not exhaustive. However, applications of Koch's postulates established that the vascular wilt of carnation in the fields sampled was caused by *F. o.* f. sp. *dianthi*. *Fusarium avenaceum* was not found to cause vascular wilt of carnation, although it was pathogenic and is reported to cause Fusarium stem and bud rot (Nelson, 1960; Baker and Tammen, 1954). It was isolated from plants that also carried *F. o. dianthi* and the conclusion reached was that the two pathogens could infect the same plant.

Some symptoms of Fusarium stem and bud rot could be confused with those of Fusarium wilt. Baker et al. (1985) describes some of the Fusarium stem and bud rot symptoms as being unilateral due to branch rot. The rotted tissue becomes reddish brown and the vascular system may get a brown discoloration. This vascular discoloration is however confined to rotted portions or areas immediately adjacent to it, unlike in the Fusarium wilt where it is widespread. This symptom similarity of the two diseases may have lead to an overestimation of the apparent vascular wilt disease severity in the fields surveyed.

The gross fungal morphology of the different isolates corresponded well with what is reported of the three *Fusarium* spp by Nelson et al. (1983), although they do not mention mesoconidia. They do, however, mention 'Macroconidia spindle-shaped and produced on monophialides or polyphialides in the aerial mycelium', a description that fits Pascoe's (1990) description of what he termed as mesoconidia. In this study, the object was not to study conidiogenesis. As such, reference to presence of mesoconidia was based purely on the presence of multi-septate, (>2) apedicellate, spindle-shaped conidia. The production of mesoconidia is favoured by culturing in a weak nutrient environment, such as Carnation Leaf Agar (CLA) (Pascoe, 1990b). CLA is recommended for growing different isolates for morphology studies (Fisher et al., 1982). This study used one-half strength PDA as it was found that the cultures grew well in it and produced conidia of acceptable uniformity and typical morphology. Identifications were also checked by National Identification Service Agriculture Canada, Ottawa. One of the *Fusarium* species is *Fusarium avenaceum* is reported to produce mesoconidia (Pascoe, 1990a) Conidia that fit the description for mesoconidia were seen on squash mounts prepared from the *F. oysporum* cultures as well from those of *F. avenaceum*. In their pictorial display of *F. oxysporum*, Nelson et al. (1983) depict some microconidia having as many as three septa. Pascoe (1990a) describes microconidia as having 0 - 1 septa. This would then seem to indicate the presence of mesoconidia.

The presence of mesoconidia has epidemiological implications because they are dry conidia and have been shown to be dispersed by air currents (Pascoe, 1990). Their production is favoured by dry, well lit conditions, and could therefore be expected to occur in above ground parts of infected plant tissues during dry seasons (Pascoe, 1990b). This would imply that it is inappropriate to regard all *Fusarium* species as exclusively splash/water dispersed soil inhabiting organisms. Tramier (1987) found a significant spread of *F. o. dianthi* by air currents in a greenhouse situation.

The isolates of *F. o. dianthi* induced significantly different K_{max} levels under greenhouse conditions. Although these could all be isolates of one race of *F. o. dianthi.* (Demmink et al., 1989), the difference between K_{max} of Isolate B (92.5%) and Isolate F (20%), is great enough to suggest that the two sets of isolates have different virulence patterns. This could imply presence of more than one race of the pathogen (Armstrong and Armstrong, 1974). Race 2 of *F. o. dianthi* is the only *F. o. dianthi.* known to occur worldwide (Baayen et al., 1988). It is also the most common race in Dutch carnation culture (Baayen et al., 1988), and the only race reported in Israel (Manicom et al.,1990). Carnation parent stock is imported into Kenya is of Dutch and Israeli origin (Hort. Co-op. Dev. Agency, Kenya. Personal comm.) and it is likely that the race of *F. o. dianthi.* found in Kenyan carnation fields would be race 2. The differences in pathogenesis among the isolates (isolates E and B induced midrib chlorosis before wilt set in. Isolates G and F induced a lot more chlorosis and less wilt symptoms) also indicates presence of more than one race (Baayen et al., 1988). Isolate H was intermediate between isolates B and E on one hand and F and G on the other. In the absence of genetically pure differential hosts, it was difficult to determine whether it comprised a different race, or if the five isolates comprised one variable race complex (Baayen et al., 1988).

Isolate E was not significantly different from isolate B. Isolate E was found to be contaminated with another fungus, possibly of *Acremonium* spp. by the National Identification Service, Agriculture Canada, Ottawa The contaminant was, however, not reisolated from carnation plants inoculated with isolate E, suggesting that if it was present, it was not pathogenic to carnation. However, we did not observe any contamination of Isolate E in our study, and the culture may have been contaminated on transit to National Identification Service. Isolates G and F did not seem to be different from one another. Their only difference in culture was that isolate F produced sclerotia and G did not. This could have been due to a mutation as *Fusarium* spp have a very high capacity for rapid mutations (Booth, 1971).

The amount of time that elapsed before onset of apparent symptoms was different for different isolates. Isolates B and E both showed symptoms by 31 days after inoculation (DAI), isolates H and G after 52 days, and isolate F after 45 days. This is a further indication of a difference in virulence patterns of the five isolates.

The number of disease severity classes (0-4) compromised discriminative capabilities or resolution, but optimized efficiency of placement of diseased plants into specific classes. The logistic model used to linearize the disease progression curve was used empirically and not biologically. Biologically, it is a model describing polycyclic disease epidemics (Vanderplank, 1963). In this work it was used to describe the disease progress of a monocyclic disease because it had the best 'goodness-of-fit' for the set of data, when compared with both monomolecular and Gompartz models. The statistics used for this comparison were the coefficient of determination, R^2 which for linear models :

$$R^2 = 1 - \frac{SSE}{SST} = \frac{SSR}{SST}$$

where SSE = Sums of squares of error

SSR = Sums of squares of regresssion

SST = Total sums of squares.

Large R^2 values (close to 1) suggest a low variability about line of predicted values (Campbell and Madden, 1990).

A further check for goodness of fit is by use of residuals which is the difference between observed values and predicted values of disease. It is therefore a measure of error ($e_i = Y_i - \hat{Y}_i$). When each residual is divided by its standard deviation, the result is standardized residuals. An appropriate model should produce a random scatter of points around predicted line when standard residuals should be randomly distributed about e = 0. When residuals are normally distributed as they should be most standard residuals will be between -2 and 2 making it easy to identify unusually large differences between observed and predicted dependent variables (Campbell and Madden, 1990).

The residual plots for the five isolates show that there were no unusually large differences between observed and predicted values

(Fig II-17). The random scatter of points around e = 0 is not immediately apparent as the number of observations are few. However, the residuals of isolates, E and F do have an unacceptable wavy pattern. Isolate E and F were best described by the monomolecular model ($R^2 = 0.981$ and $R^2 = 0.824$ respectively). This was compromised in the search of one common model that best described all of the disease progression curves of all five isolates.

The application of a disease progression model in this work assumed a maximum level of disease (K_{max}) of 100% or 1.0. This was not a valid assumption and there was, therefore, a general underestimation of the rate parameters, r_L (Neher and Campbell, 1992). A solution to this problem would have been the use of non - linear regression analysis. However, this would have required a minimum of nine observations through time to enable convergence of the model for the empirical data. Observations over time in this work were too few.

Thus, it could be concluded that:

i) carnation wilt in Kenyan fields is caused by a mixture of both *F. o. dianthi.* and *F. avenaceum*;

ii) it is possible that there are more than one races of *F. o. dianthi.* infecting the carnation and one of them could possibly be race 2.

Further studies on the race identification using genetically pure differential hosts need to be carried out. This however is time consuming; requires extensive experimentation facilities. Two methods for rapid identification of pathogenic isolates have been suggested by Manicom et al (1990). In their work, they report on use of restriction fragment length polymorphisms (RFLP) and vegetative compatibility groups (VCG) for the genetic determination of races and their relation to pathogenicity for a collection of *F. o. dianthi* isolates.

F. Tables, Figures, and Legends.

Table II-1 Summary of linear regression statistics†† used in the description of disease progress of carnation Fusarium wilt caused by five isolates of *Fusarium oxysporum* f. sp. *dianthi*·

ISOLATE	R^2	Y INTERCEPT	R_L
B	0.993	-5.952	0.106
E	0.915	-4.512	0.074
H	0.969	-5.114	0.066
F	0.655	-5.188	0.05
G	0.904	-5.483	0.049

R^2 = coeffecient of determination. R_L = slope parameter.

††when data was fitted to the logistic model.

Note that only disease severity proportion values greater than 0 and less than 100 were used.

Table II-2 Analysis of variance of disease indices at K_{max} [*] for five isolates of *Fusarium oxysporum* f. sp. *dianthi.*

ISOLATE	DISEASE INDEX MEAN[†]	F VALUE
B	3.7^{a}	$10.77^{\S}$
E	3.3^{a}	
H	2.2^{ab}	
G	0.8^{b}	
F	0.8^{b}	

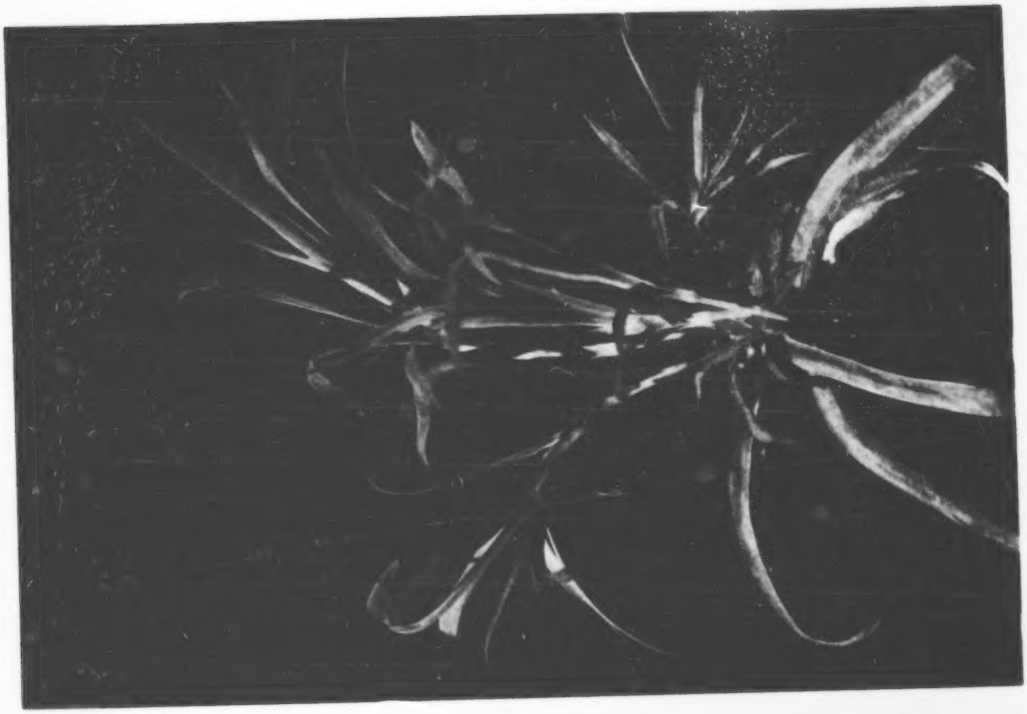
[†] Means from ten replicates. Means ending with the same letter are not significantly different by Student-Newman-Keuls (SNK) test at alpha = 0.01.

[*] K_{max} = maximum level of disease attained during the disease epidemic.

[§] Computed F is significant at alpha = 0.01.

Fig. II-0. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of zero

Fig. II-1. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of one



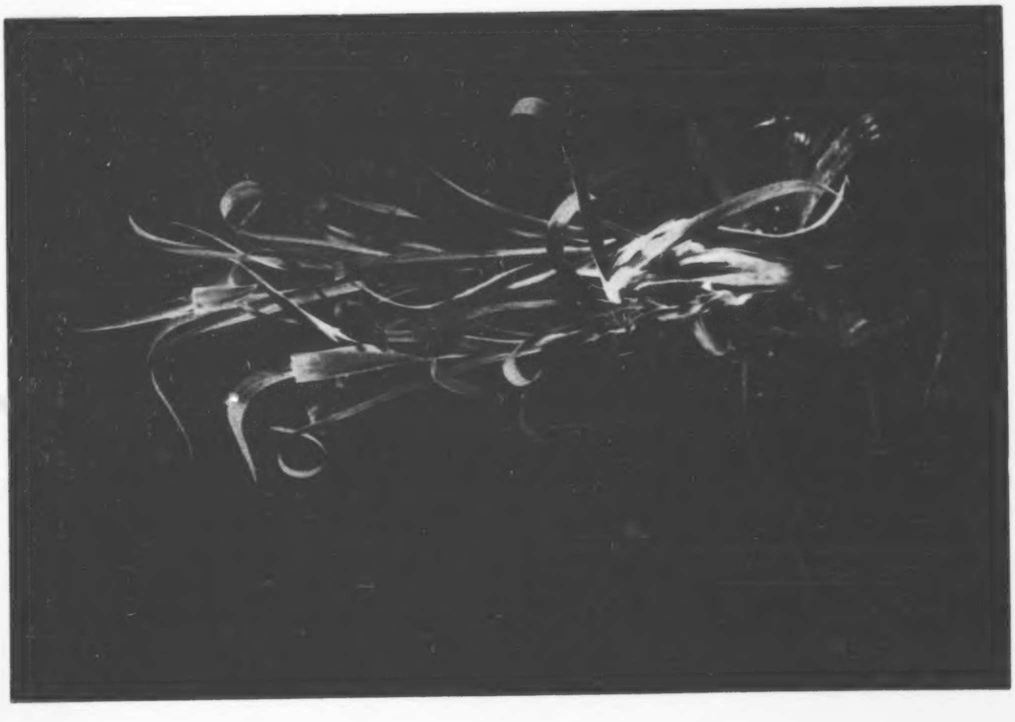


Fig. II-2. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of two

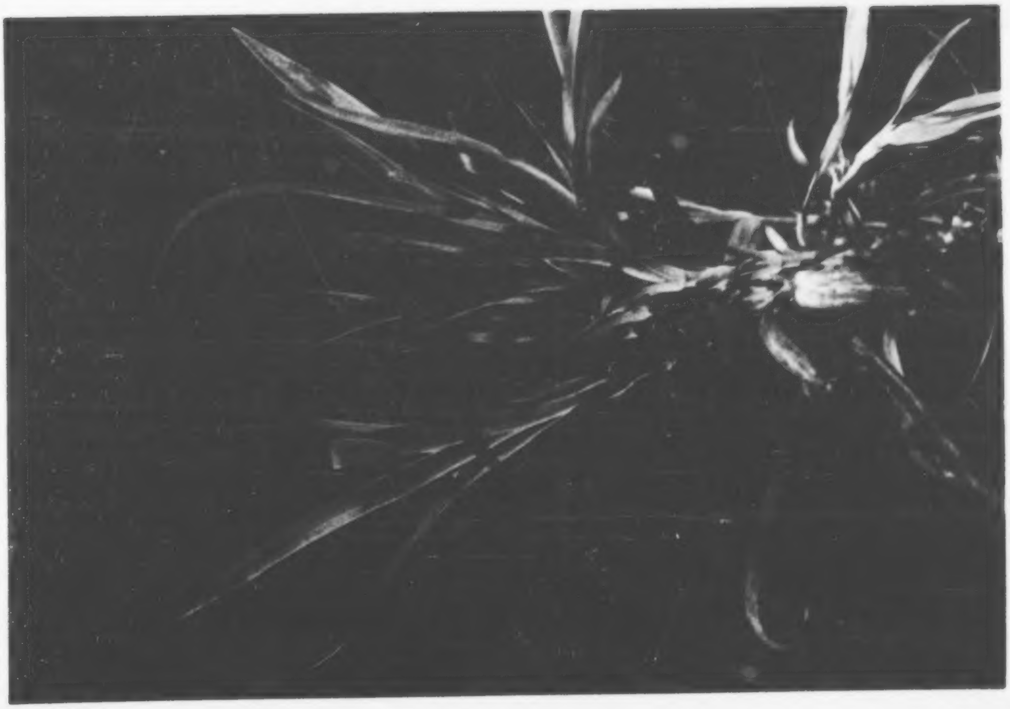


Fig. II-3. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of three

Fig. II-4. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of three

Fig. II-5. Carnation plant infected with *F. o. dianthi* (isolate B), showing a disease index of four

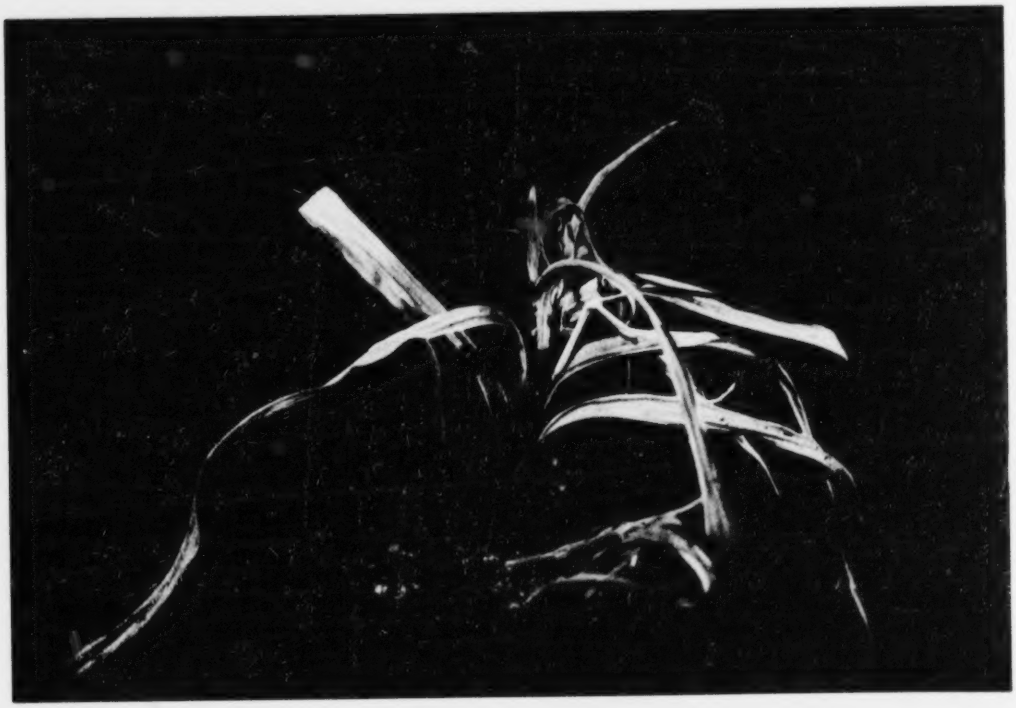


Fig. II-6. Fourteen-day-old cultures of fungal isolates from wilted carnation plants.

W All the eight isolates grown on one petri plate

X Colony reverse of W

Y *F. equiseti*

Z Colony reverse of Y

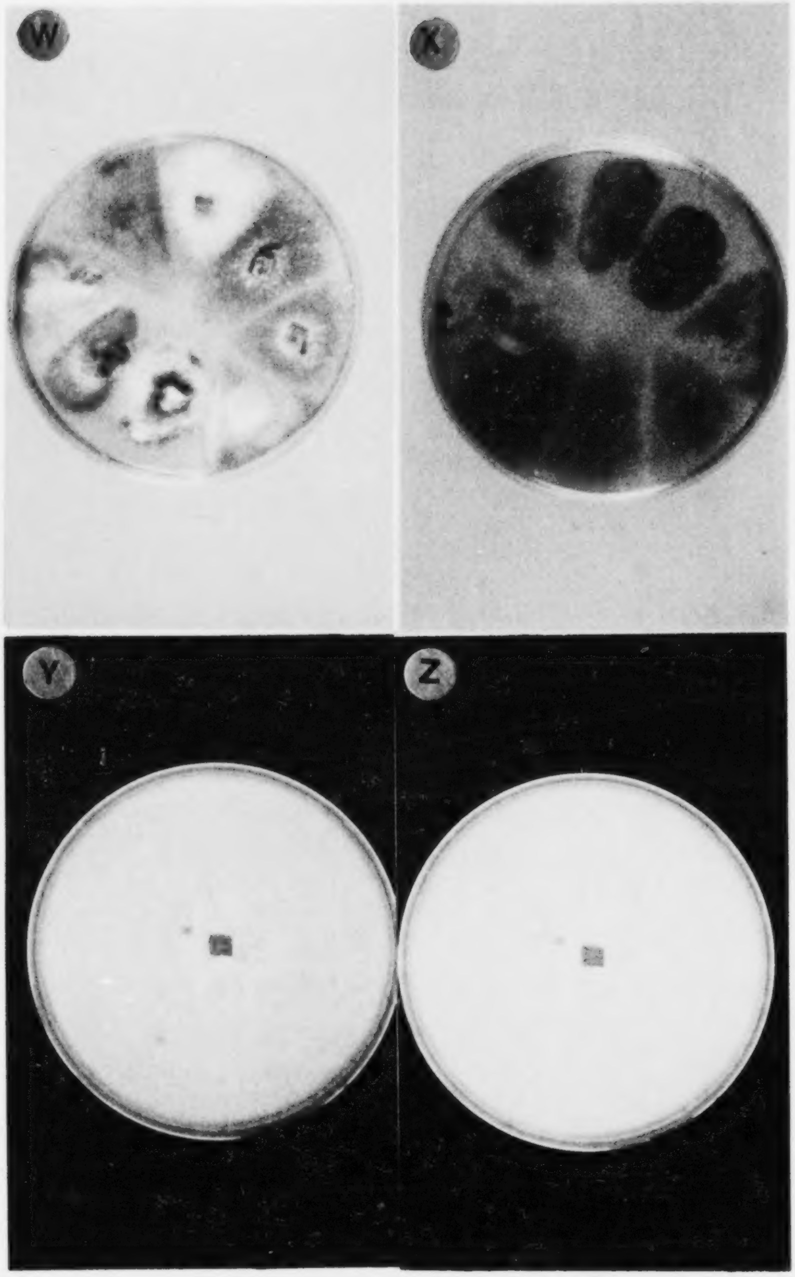

W
X
Y
Z

Fig. II-7. Fourteen-day-old cultures of *Fusarium avenaceum* isolates from wilted carnation plants

W Isolate C

X Colony reverse side of W

Y Isolate D

Z Colony reverse of Y

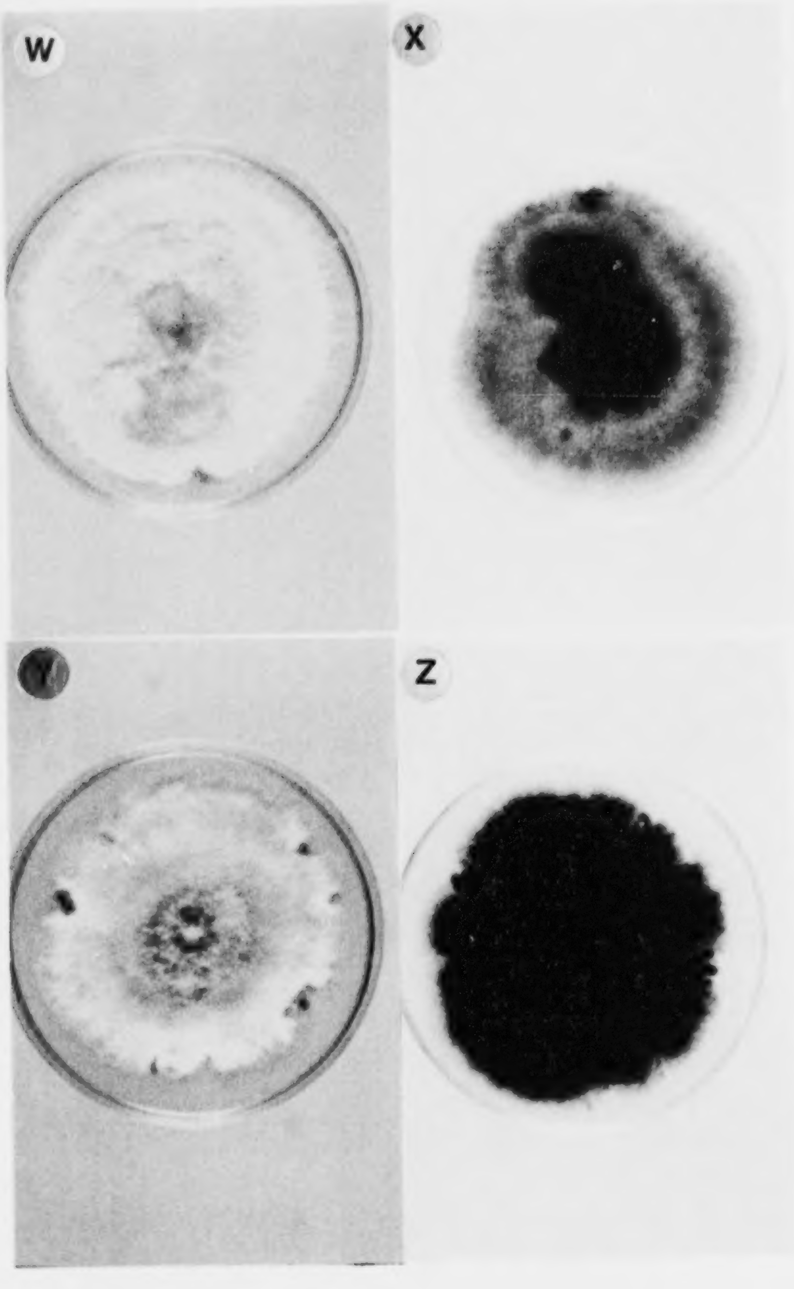

W
X
Y
Z

Fig. II-8. Fourteen-day-old cultures of *Fusarium oxysporum* isolates from wilted carnation plants

W Isolate B

X Reverse colony of W

Y Isolate E

Z Reverse colony of Y

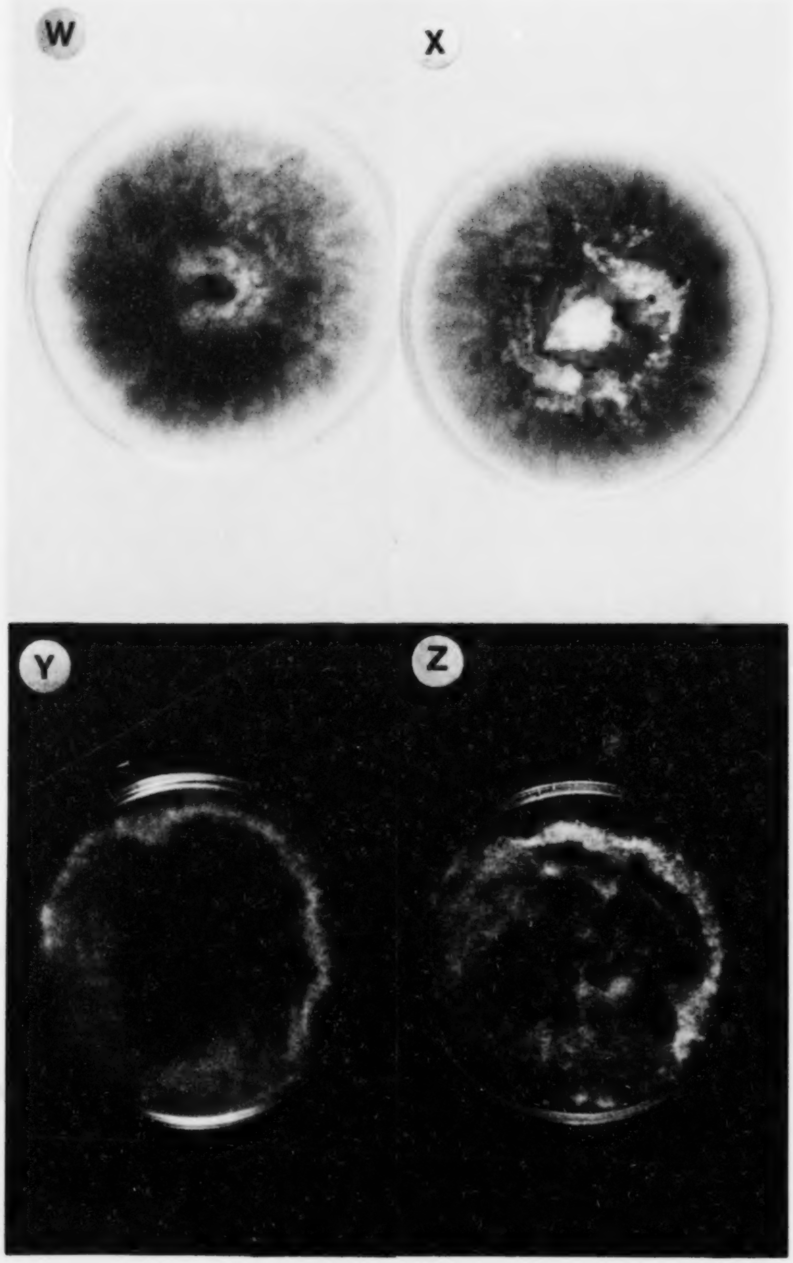

W
X
Y
Z

Fig. II-9. Fourteen-day-old cultures of *F. oxysporum* isolates from wilted carnation plants

W Isolate F
X Colony reverse of W
Y Isolate G
Z Colony reverse of Y

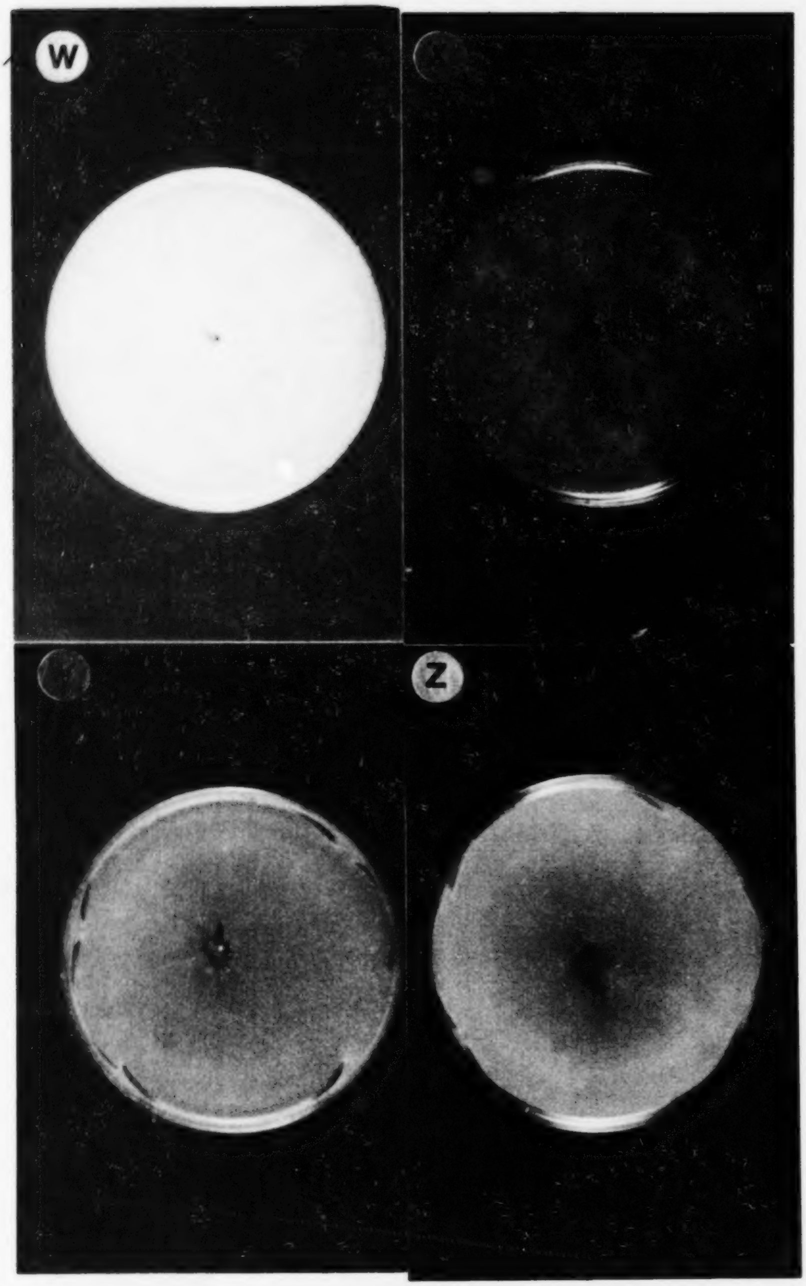

W
Z

Fig. II-10. Fourteen-day-old cultures of *F. oxysporum* isolates from wilted carnation plants

W Isolate H

X Colony reverse of W

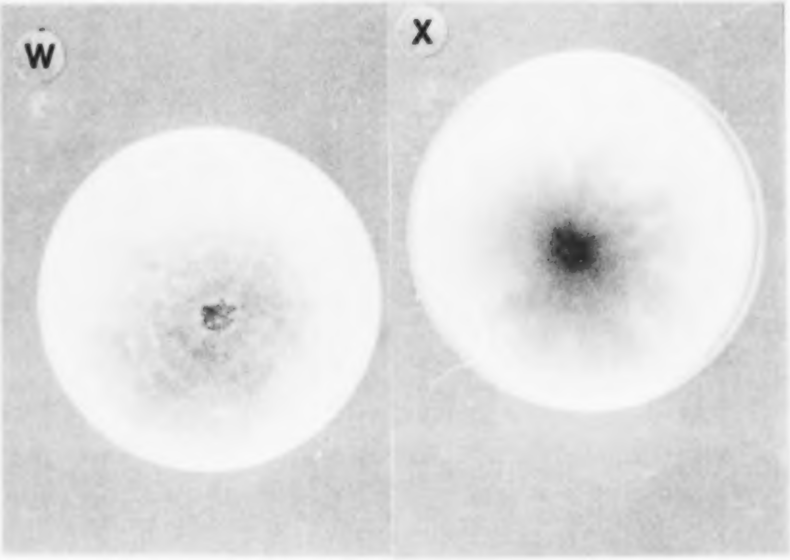

W
X

Fig. II-11. Macroconidia , microconidia and chlamydospores of *Fusarium equiseti* (× 500)

W Chlamydospores

X Macroconidia

Y Microconidia on a microconidiophore

Z All of the above

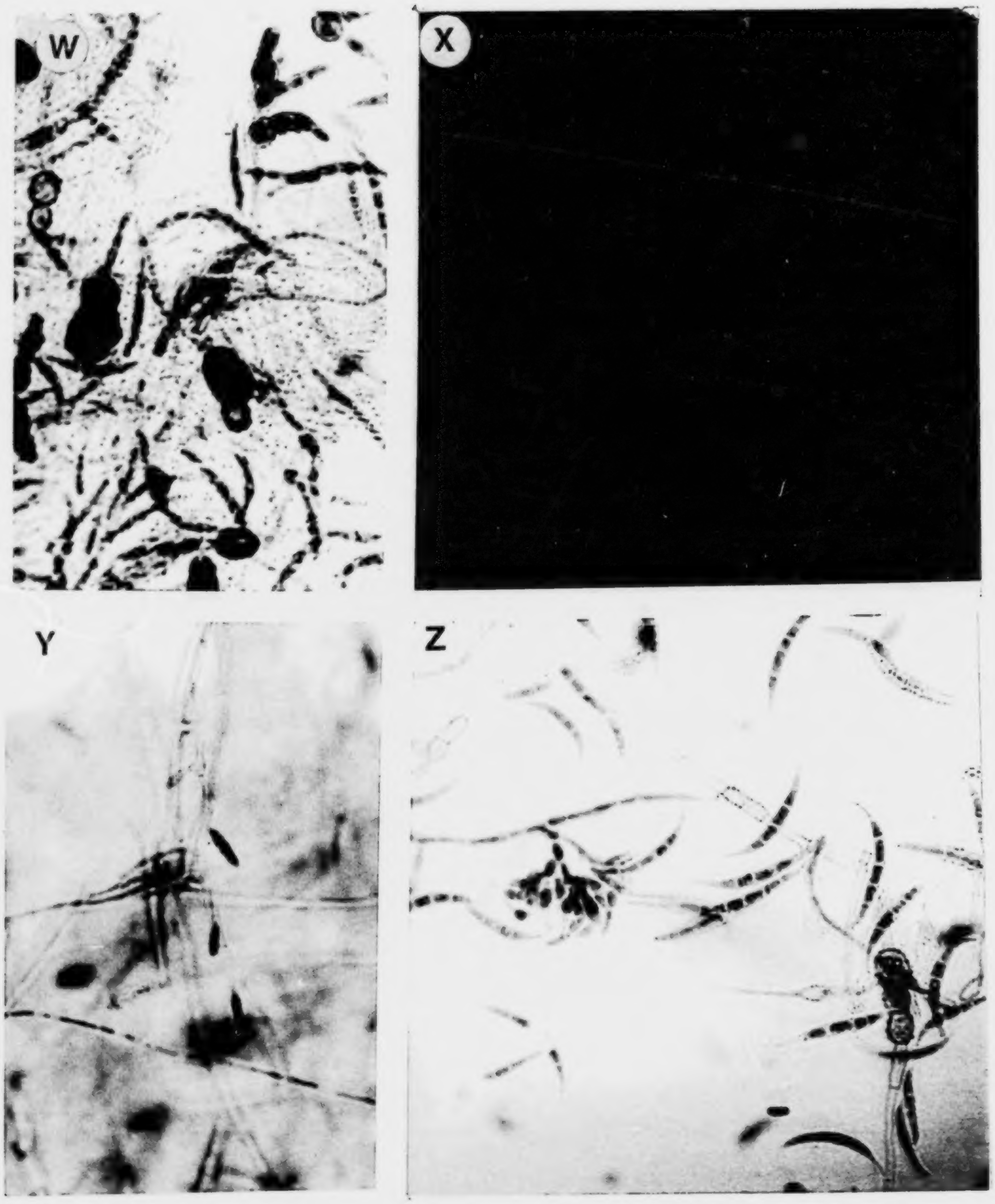

W
X
Y
Z

Fig. II-12. Macroconidia and microconidia of *Fusarium avenaceum* (× 500)

W and X Branched and unbranched monophialides

Y Macroconidia

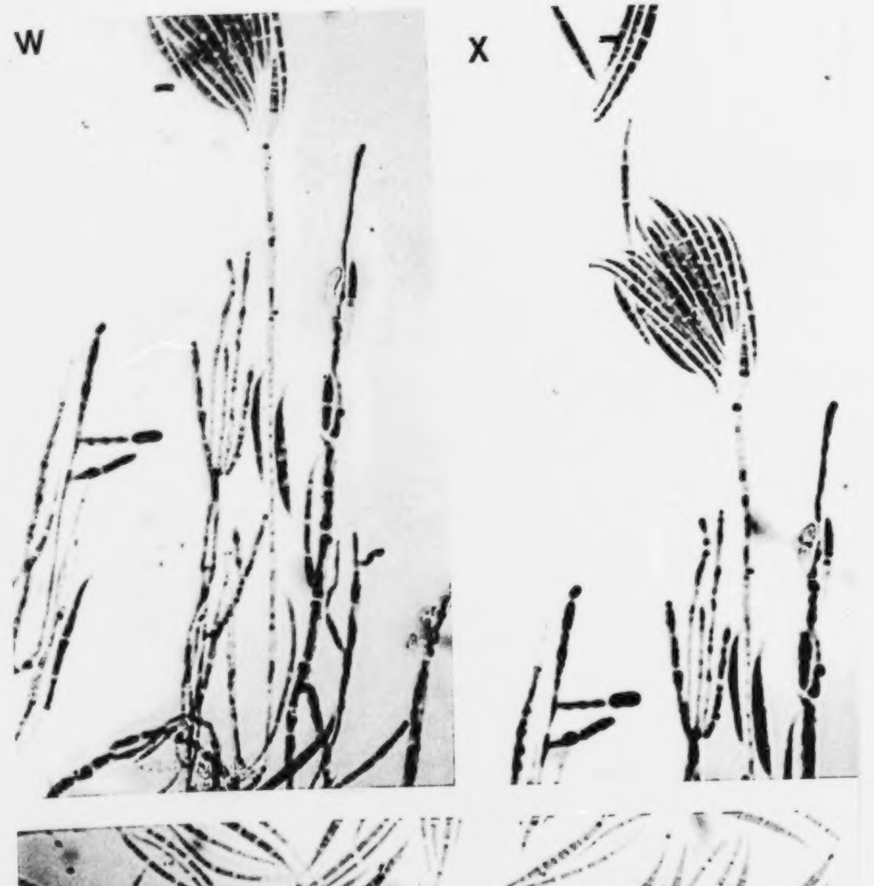

W
X
Y

Fig. II-13. Macroconidia , microconidia and chlamydospores of *Fusarium oxysporum* (× 500)

W Macroconidia

X Macroconidiophore

Y Chlamydospores forming in conidia

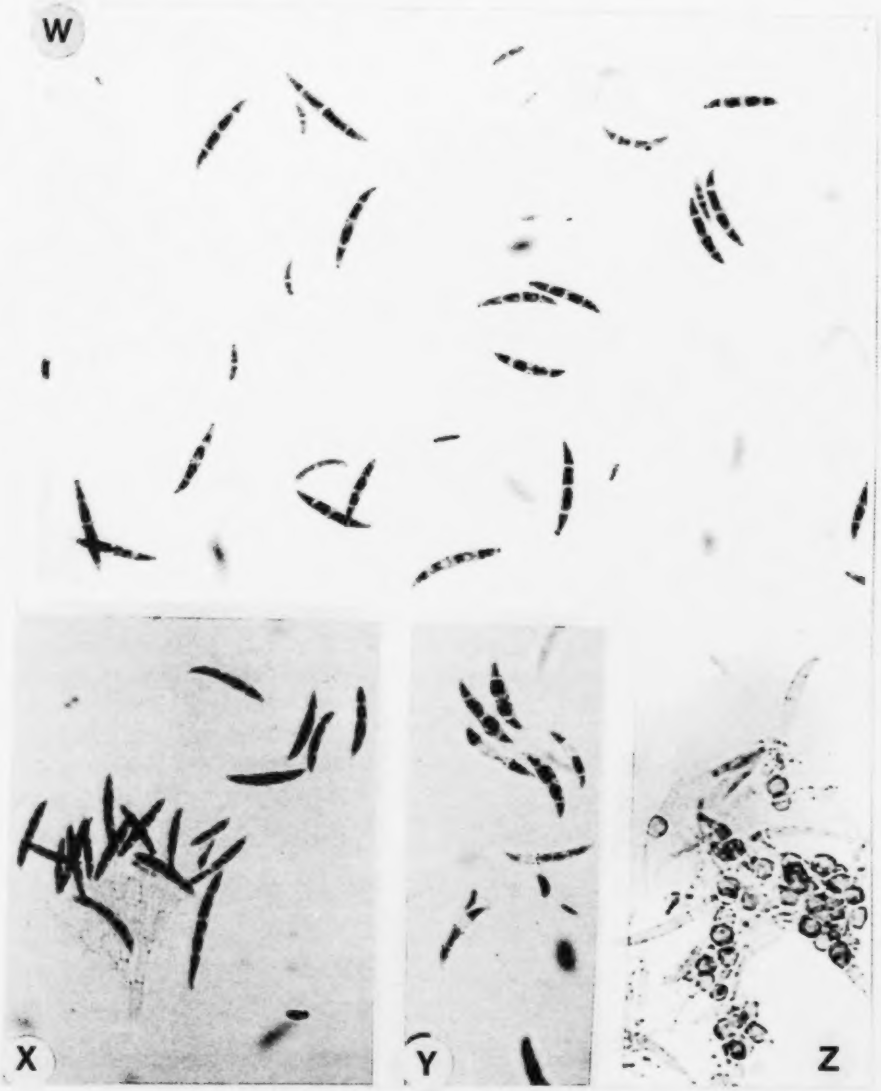

W
X
Y
Z

Fig. II-14. Six-week-old carnation transplant inoculated with *F. avenaceum*

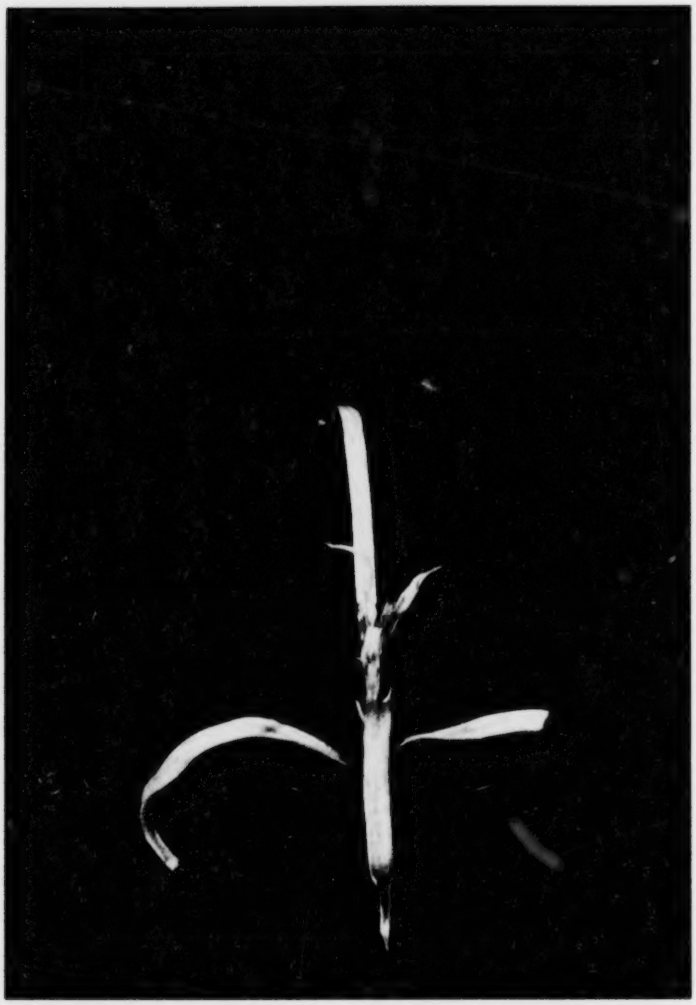
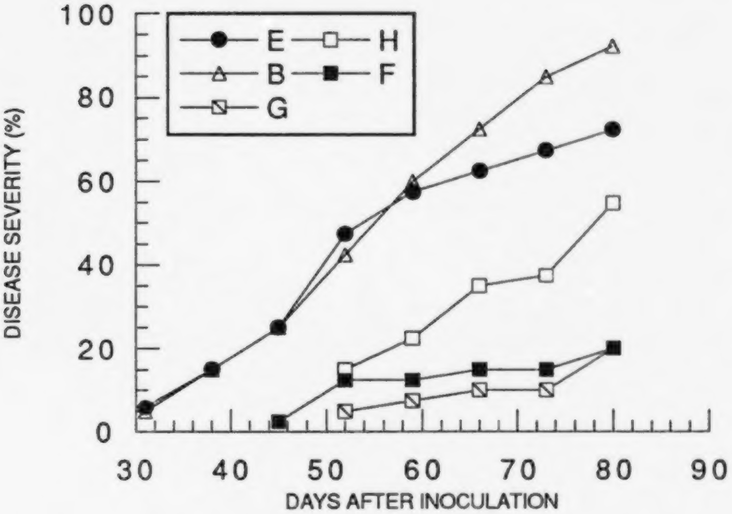


Fig. II-15. Six-week-old carnation transplant inoculated with *F. avenaceum* compared with a healthy plant



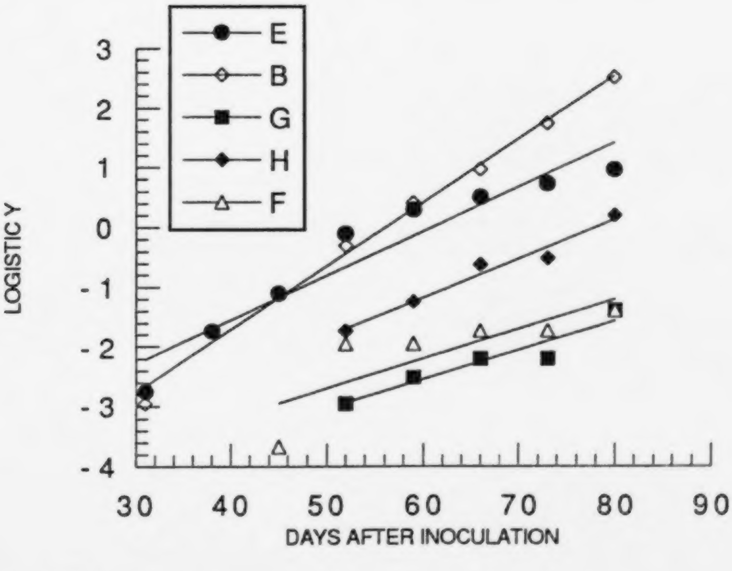


Fig. II-16 Actual and predicted Fusarium wilt disease severity values plotted against time for carnation cv. Portrait and five isolates of *Fusarium oxysporum* f. sp. *dianthi*.

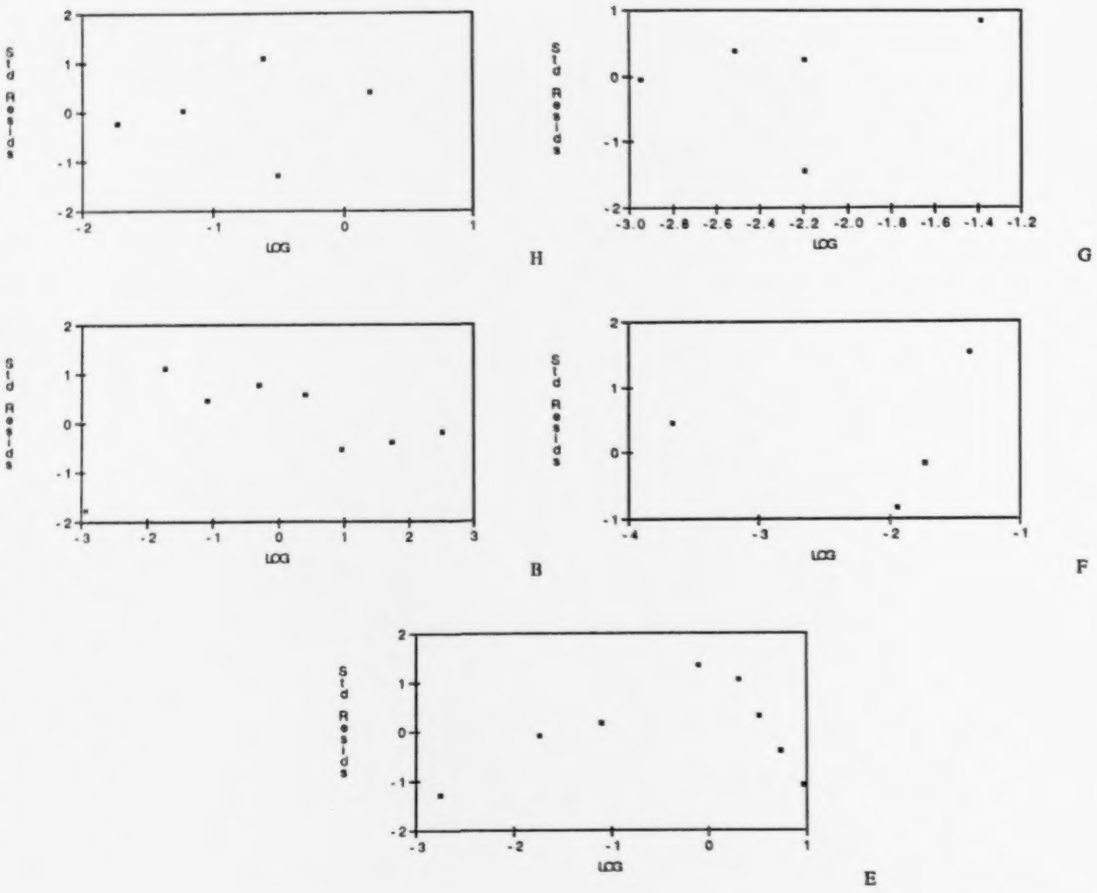


Fig. II-17. Standardized residuals plotted against logits (transformed Y) from linear regression analysis, to evaluate appropriateness of the logistic model for describing epidemics of vascular wilt caused by five *F. oxysporum* isolates.

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Chapter III

Interaction of Fusarium Wilt of Carnation With VAM Fungi

A. Introduction

Carnation plants infected with *Fusarium oxysporum* f. sp. *dianthi* develop chlorotic leaves, crook neck shoots and eventually unilateral wilt (Pennypacker and Nelson, 1972). Internally, the carnation plants respond to the infection with vascular plugging, hypertrophy and hyperplasia of xylem parenchyma cells, vascular regeneration, differentiation of pith (medulla) parenchyma into xylem vessel elements and, xylem parenchyma cell disintegration, leading to formation of vascular cavities (Pennypacker and Nelson, 1972; Baayen, 1986, 1988; Harling and Taylor, 1985). These are all normal carnation defence reactions to fungal invasion In histopathological studies involving susceptible and resistant carnation cultivars. Baayen and Elgersma (1985) observed that in susceptible cultivars, stems were intensively colonized and vessels were rarely occluded with gels. They also found that cell degradation led to the formation of stem cavities and they did not observe any hyperplasia of xylem parenchyma. Colonization in the resistant cultivar remained low throughout their experiment, but they observed vascular gelation which they considered to be a part of the resistance mechanism of carnation to Fusarium wilt, and hyperplasia of xylem parenchyma bordering infection. Xylem regeneration was observed following the gelation. This type of regeneration was been observed previously (Harling et al. (1984), and had been associated with vascular gelation and plant cell degeneration by the fungus. In later studies using dye translocation (Baayen, 1986), hyperplasia and vascular tissue regeneration were observed to compensate for local vascular dysfunction (caused by occlusion and degradation) for water transport. From this study, Baayen (1986) concluded that vascular regeneration in carnation is a normal defence reaction to fungal invasion.

At present, there is no single measure that fully controls Fusarium wilt of carnation. An integration of different control measures permit management of the disease level (Garibaldi and Gullino, 1987). Since carnation culture uses vegetative cuttings for propagation, preinoculation of the cuttings with vesicular-arbuscular mycorrhizal (VAM) fungi is feasible. Should inoculation with VAM fungi suppress disease severity of Fusarium wilt, it will provide a further means of disease control that can be easily integrated into existing cultural practices. Vesicular-arbuscular fungi also have the potential to overcome the disadvantages of soil fumigation by replacing it in disease control (Nemec, 1987). The objectives of this study were therefore to:

1. carry out a histological study on stems of resistant and susceptible carnations infected with *F. o. dianthi*.

2. assess the effect of VAM fungi on plant dry weights, phosphorous levels of aerial part of the plant, and severity of Fusarium wilt caused by *F. o. dianthi*

B. Materials and Methods

A series of experiments were set up to study the effects of VAM fungi on carnation, and on Fusarium wilt in carnation.

1. Infection of Carnation by VAM

Rooted cuttings of carnation cv. Lolita were obtained from Yoder Carnation (Yoder Canada,. Leamington, Ontario). Cultures of three VAM species; *Glomus intraradices* Schenck and Smith, *G. mosseae* (Nicol. and Gerd.) Gerdmann and Trappe, and *G. dimorphicum* Boyetchko and Tewari, were multiplied on alfalfa in steam sterilized sand:soil (3:1) mix. Inocula of *G. intraradices* and *G. mosseae* were originally obtained from Dr. N. C. Schenck by Dr. J. P. Tewari as International Culture Collection of VA Mycorrhizal Fungi (INVAM), University of Florida, Gainsville #208 and 156, respectively. Inoculum of *G. dimorphicum* used was from the type culture of this species (Boyetchko and Tewari, 1986). Alfalfa was maintained in the greenhouse in 15 cm pots at 24 C day and 20 C night temperature regime, 16 hr photo period and a maximum light intensity of 500 $\mu E\ m^{-2}\ s^{-1}$. The sand:loam mix had an initial available phosphorous level of 5.9% (Agric. Service Laboratory Inc., Kitchener, Ontario). The VAM inoculated alfalfa was planted in 15 cm pots. Full-strength Hoagland solution minus phosphorous and adjusted to pH 6.0 was used as fertilizer at 100 ml pot^{-1} $week^{-1}$, with regular watering.

After five months, the pot cultures were harvested, roots were removed from the soil and the number of VAM spores per g of soil was determined for every species of VAM. The sucrose gradient centrifugation method of spore extraction (Ianson and Allen, 1986) was used on 10 g soil samples. The extracted spores were counted and number of spores g^{-1} of soil was determined. The spore concentration in inoculum of each VAM species, was then adjusted to 100 spores g^{-1} of soil by dilution using steam sterilized sand : loam (3:1) mix. This was the functional inoculum.

Rooted carnation cuttings were planted in a 3:1 sand:loam mix in 15 cm pots, one plant pot^{-1}. Five g of VAM inoculum was placed just below but not in contact with the carnation roots. One carnation cultivar, Lolita was used with four VAM treatments i.e. no VAM, *G. intraradices*, *G. dimorphicum*, and *G. mosseae*. Each potted plant was an experimental unit and each treatment had twenty replicates. The pots were placed in a growth cabinet in a completely randomised design. Day/night temperatures were maintained at 24 C / 20 C night with a 16 hr photoperiod and a maximum light intensity of 350 μE m^{-2} s^{-1}. The fertilizer regime was as described for the multiplication of VAM fungi.

Plants were sampled for VAM-infection assay at 42 and at 90 days after inoculation (DAI). A random sample of three plants was taken from every treatment for each assay period. Roots of each plant were washed in tap water, then cleared and stained as described by Phillips and Hayman (1970). The roots were first heated in a 90 C oven for one hour in 10% potassium hydroxide. The KOH was then poured off and roots were rinsed in tap water until no brown color appeared in the rinse water. Rinsed roots were bleached in alkaline hydrogen peroxide (3 ml ammonium hydroxide, 30 ml 10% hydrogen peroxide, 567 ml tap water) for ten minutes, rinsed in water and acidified in 1% trypan blue lactic acid staining solution (875 ml lactic acid, 63 ml glycerine, 63 ml tap water, 0.1 g trypan blue) for five minutes. The staining solution was then poured off and lactic acid destaining solution (same as the staining solution minus trypan blue) was used to remove the excess stain.

Percent root colonization as described by Giovannetti and Mosse (1980) was determined using the grid-line intersect method. This is a modification (Giovannetti and Mosse, 1980) of the line transect method developed by Newman (1966). Cleared stained roots were placed in areas measuring 1.0 × 2.0 inch on microscope slides, covering as much of these areas as possible but not overlapping. The slides containing root samples were then placed onto a petri dish that was marked with grid lines forming 0.5 in^2. The grid lines thus marked corresponded to systematic observation points. An estimate of the root length was given by:

$$RL = \frac{\pi N A}{2 H} \quad \text{where,}$$

N = number of intersections,

A = area over which root segments are spread,

H = total length of horizontal and vertical grid lines per slide.

At any particular position along the root segment, the intersections were scored for presence and absence of VAM. Macerated roots and roots whose cortex was missing were not included in the assessment. The percent root infection was determined by dividing the total mycorrhizal root length by the total length of root per sample. Observations were made under a dissecting microscope at magnifications up to ×50, except when need for a more detailed observation necessitated use of a compound microscope.

2. Effect of VAM Fungi on Foliar Phosphorous Content and Dry Weight of Carnation

A 3 × 2 × 20 factorial experiment was set up using three carnation cultivars and two levels of VAM. The cultivars were Portrait, Scania and Lavender Lace. They were chosen on the basis of their levels of resistance to Fusarium wilt as classified by Yoder Carnation. Portrait, Scania, and Lavender Lace are susceptible, moderately resistant, and resistant, respectively. The VAM fungus used was Nutrilink (NPI Inc, Salt Lake City, Utah), a commercial grade *G. intraradices*, containing 1000 spores g^{-1} of inoculum holding medium. A completely randomized experimental design was used. Each cultivar × VAM combination was a treatment. Each plant constituted an experimental unit. VAM inoculum levels were 0 and 5 g pot^{-1}.

Sixty-two days after inoculation, at the onset of flowering, five plants were randomly selected from every treatment. The aerial portion of each plant was oven dried at 70 C for 72 hr. The dry weights were recorded and put through analysis of variance procedures on SAS (SAS Inst. Inc. Cary, North Carolina) for mean separations. The dry plant material samples were then sent to Agri-Service-Laboratory Inc., Kitchener, Ontario for analysis of %

phosphorous content. The means were then separated using the Student-Newman-Keuls test.

3. Effect of VAM Fungi on Fusarium Wilt of Carnation

This was a 3 × 4 × 2 × 10 factorial experiment. The same three carnation cultivars were used. The four VAM treatments used were; *G fasciculatum*, (obtained from Dr. N. R. Knowles, Dept. Pl. Sc. University of Alberta) *G. intraradices*, *G. mosseae*, and no VAM. Inoculation with *F. o. dianthi* was at two levels; 0 and 7.2×10^7 colony forming units (CFU). Carnation cuttings were obtained from Yoder Carnations. The cuttings were rooted in a misting chamber with an ambient temperature of 20 C day and 18 C night. Bottom heat at 22 C was used.

The rooting medium used was perlite underlaid with sphagnum moss. The VAM inoculum was incorporated and thoroughly mixed into the rooting medium to give a concentration of 100 spores g^{-1}. For the uninoculated controls, the rooting medium was mixed with a sand:loam (3:1) mix that had been double steam sterilized. A light intensity of 250 $\mu E\ m^{-2}\ s^{-1}$ was used during the first few days after the cuttings were inserted into the rooting medium. Thereafter, a light intensity of 500 $\mu E\ m^{-2}\ s^{-1}$ was maintained. A 12 hr photoperiod was maintained throughout the rooting process.

At the end of six weeks, three rooted cuttings from every treatment were randomly selected. The roots were cleared, stained, and scored for % VAM infection as previously described. At the same time, twenty rooted cuttings were selected from each treatment and potted in 10 cm pots. Half of the plants were then inoculated with 7.2 × 10^7 CFU of *F. o. dianthi* isolate B. The remaining half were used as controls. The inoculation process was as described in chapter two. The pots were placed on benches in the greenhouse using a completely randomised block design. The blocking was by carnation cultivar. Progression of Fusarium wilt was monitored. All plants were fertilized once a week with 100 ml pot^{-1} of full strength Hoaglands solution minus phosphorous. Temperature was maintained at 23 C day and 20 C night with a 16 hr photoperiod and a light intensity of 500 $\mu E\ m^{-2}\ s^{-1}$.

Disease progression curves were constructed and the slopes compared by regressing transformed disease proportions against time. The model that best fitted the data set was Gompartz, with the following linearized equation (Campbell and Madden, 1990):

$$Y_G = -LN[-LN(Y_o)] + r_G t = -LN[-LN(Y)],$$

where:

Y_G = transformed disease proportion,

Y_o = initial disease level assuming epidemic starts at $t = 0$,

r_G = Gompartz rate parameter,

Y = non-transformed disease proportion,

t = time.

The disease indices of every treatment at k_{max} were put through ANOVA procedures to check for any significant differences in maximum disease severity attained using each treatment.

4. Histopathology of Carnation Infected With *F. o. dianthi*

Sixty DAI with *F. o. dianthi*, stem samples were taken from infected plants in every treatment. Sampling was done at three sections of the stem; the base, midway between base and tip (3^{rd} and 4^{th} internodes), and at the tip. The stem pieces were immediately fixed in 3% glutaraldehyde in distilled water overnight at room temperature, in preparation for scanning electron microscope (SEM) studies as described by Goldstein et al., (1981). The fixed samples were cut into 6 and 3 mm long blocks. Long sections were cut from the 6 mm blocks and cross sections from the 3 mm long blocks. The sections were then washed in distilled water and post fixed in 2% osmium tetroxide in distilled water overnight, after which they were again washed in three 30 min changes in distilled water. They were then subjected to 15 min dehydration in each of 15, 30, 50, 70, and 95% ethanol. This was followed by a 30 min change in 1:1 mixture of amyl acetate and 95% ethanol and finally the sections were placed into pure amyl acetate ready for critical point drying in a CPD Polaron Jumbo. After critical point drying, the samples were mounted on specimen holders and gold coated on the days they were examined in the SEM microscope (SEM Cambridge S250). They were examined for the presence of *F. o. dianthi* in stem tissues and for any morphological/physiological changes of the plant cells due to disease. X-ray microanalysis was also done using the X-ray Tracor Northern 5500.

C. Results

1. Infection of Carnation by VAM

Both *G. intraradices* and *G. mosseae* infected the roots of Lolita and produced vesicles and arbuscules, but there was no visible infection of the roots by *G. dimorphicum*. The grid-line intersect method did not detect any VAM infection in the roots 30 DAI, although a few points of infection and some arbuscules were observed in *G. intraradices* infected roots. At 42 DAI, the percentage infection of roots by *G. intraradices* was 7.7%. This was slightly higher than 6.6% infection by *G. mosseae* at this stage. At ninety DAI percentage root infection by *G. mosseae* (27.1%) was more than double of that by *G. intraradices* (10.9%) (Fig. III-0). The roots of plants inoculated with *G. mosseae* as well as those of plants infected with *G. intraradices* were seen to be infected by *Rhizoctonia* sp. which formed masses of microsclerotia within the plant cells (Fig. III-1). In addition to *Rhizoctonia* infection, roots inoculated with *G. intraradices* were also infected by *Olpidium brassicae* (Wor.) Dang. (Fig. III-1) and an organism that had resting spores strongly resembling those of *Lagena radicicola*. The organism was in the form of ellipsoid bodies that were 12 × 21 μm (only ten spores measured) with walls that were about 1 μm thick. This organism was also seen to parasitize the VAM vesicles in the roots (Fig III-2). The organism seemed to multiply within the vesicles and finally lyse the vesicle (Fig III-2). The controls were clean of any infection.

2. Effect of VAM Infection on Dry Weights of Carnation

Analysis of variance (Table III-1) showed that only VAM effects were significant at alpha = 0.01 (P = 0.0001). Cultivar and VAM x cultivar interaction effects were not important even at alpha = 0.05 level. The mean dry weight (MDW) of VAM inoculated plants of Lavender Lace was significantly higher than the MDW of the uninoculated controls at alpha = 0.01. In Scania, the MDW of VAM inoculated plants was only significantly higher than that of uninoculated controls at alpha = 0.05. Means of Portrait were not significantly different even at alpha = 0.05 (Table III-2).

3. Effect of VAM Infection on Available Phosphorous of Carnation

Analysis of variance showed a significant effect of VAM (PR > F = 0.004) but cultivar and interaction effects were not significant even at alpha = 0.05 (Table III-1) for the full model. However, percent phosphorous means of VAM inoculated plants were not significantly different from those of uninoculated controls at alpha = 0.01 for any of the three cultivars. The percent phosphorous mean of VAM inoculated plants were significantly lower than those of uninoculated controls at alpha = 0.05 for Portrait (Table III-3).

4. Effect of VAM Fungi on Fusarium Wilt of Carnation

The grid-line intersect method did not detect any infection of roots by any of the three VAM spp. in any of the three carnation cultivars. However, infection points and a few vesicles were observed under a compound light microscope. The vesicles were only observed in L. Lace roots infected with *G. fasciculatum* (Fig. III-3). Arbuscules were not observed.

Cultivar and VAM effects on k_{max} were significant at alpha = 0.01, but interaction effects of VAM x cultivar were not significant even at alpha = 0.05. In the no VAM treatment, the k_{max} of Lavender Lace was significantly lower than that of Scania and that of Portrait at alpha = 0.01. Plants inoculated with the three VAM fungi did not have k_{max} values that differed significantly amongst the cultivars at alpha = 0.01 (Table III-4). Disease progression curves (figs. III-4a-d) showed a general depression of Fusarium wilt in carnation with VAM inoculation, except for the L. Lace where disease level was enhanced by inoculation with all three species of VAM, and more so *G. fasciculatum* (Table III-5).

A. Lavender Lace

With no VAM inoculation, this cultivar had a significantly lower k_{max} value (Table III-4) compared to the other two cultivars. With VAM inoculation, however, this difference disappeared and the k_{max} values increased (Table III-4). The slopes of the disease progression curves became steeper with VAM inoculation, increasing from 0.02 with no VAM to 0.05 when infected with *G. fasciculatum* (Table III-5).

B. Scania

There was no significant difference on the k_{max} values with VAM infection (Table III-4). However, the VAM infection reduced the slope of the disease progression curve from 0.7 to 0.5 (Table III-5). However, the slope did not change with *G. fasciculatum* infection (Table III-5).

C. Portrait

The ANOVA procedures did not show any significant effect of VAM fungi on disease severity in this cultivar. However, regression analysis on the disease progression curve did show a flattening of slope response of disease to VAM infection (Fig. III-4d; Table III-5) Infection with *G. fasciculatum* reduced the slope from 0.06 (control) to 0.03 (Table III-5).

5. Scanning Electron Microscopy

Plants of all cultivars infected with *F. o. dianthi* were colonized both intercellularly and intracellularly (Fig. III-6). Infection was not only confined to the xylem elements, but also moved into the cambium, phloem, and pith (Fig. III-6). In the susceptible Portrait and moderately resistant Scania, infection was heavy and degradation of vascular tissue was extensive (Fig. III-7), whereas in the resistant Lavender Lace infection was much less and vascular tissue degradation was virtually lacking Hyperplastic xylem parenchyma and pith cells, regeneration of xylem vessels, and presence of deformed cells due to hyperplasia were observed. Differentiation of pith cells into pitted xylem vessels was also observed (Figs. III-6). Occlusion of xylem pits with 'droplet' plugs (Fig. III-8), occlusion of cells by fungal material, plugs of an amorphous deposit, and calcium deposits were also observed in all cultivars (Fig. III-9). The cells bordering colonized xylem had thickened walls (Fig. III-10). Sometimes the amorphous deposit material contained high amounts of calcium, (Fig. III-11), but mostly it seemed to be made up of carbohydrates (Fig. III-12). Energy dispersive x-ray microanalysis showed that this material bound high amounts of osmium, suggesting it had a high lipid content. Vascular tissue and adjacent cells of infected stems were observed to deposit calcium along cut surfaces of their walls (Figs. III-13, III-14). Calcium deposits were also observed in the cell lumens. This was observed in both susceptible and resistant cultivars, but more so in the resistant cultivar Lavender Lace. X-ray mapping of calcium in cross sections taken from the mid-stems of L. Lace and the susceptible Scania show this difference (Fig. III-15). The maps showed similar amounts of calcium towards the edges of the stems for both the infected and the control plants. The vascular and sorrounding tissues of infected plants however contained more calcium than did those of control plants (Fig. III-15). Tyloses were also observed in Scania, but only in one section (Fig. III-16).

D. Discussion

The carnation cultivar Lolita was infected by *Glomus mosseae* and *G. intraradices*, but not by *G. dimorphicum*, indicating a cultivar by VAM interaction effect. This effect was emphasized when a second experiment was carried out using three other carnation cultivars, Scania, Portrait, and Lavender Lace. None of these showed vesicle formation with either *G. mosseae* or *G. intraradices.* Lavender Lace, however did show some vesicle formation when inoculated with *G. fasciculatum.* This disparity in response to VAM among host cultivars (genotypes) within the same species is not novel and has been reported (1983; Mercy et al., 1990). Mycorrhizal colonization can affect the biochemical and physiological activities of the plant (Baltruschat and Schönbeck, 1975; Gianinazzi and Gianinazzi, 1983; Hussey and Roncadori, 1982), a response that is likely to be host-genotype dependent.

Mercy et al. (1990) described mycorrhizal colonization in cowpea to be host-dependent and heritable. Results from this study indicated a high likelihood that infection of carnation by VAM, could be genotype dependent. This indicates that it may be possible to improve carnation susceptibility through breeding. The problem here would be that selection for VAM susceptibility would have to compete with selection for floricultural characteristics. Of the four carnation cultivars inoculated with VAM, only one resulted in a relatively high level of infection, and this explains why the family Caryophyllaceae is labelled as non-mycorrhizal (Tester et al., 1987) and also shows that only some genotypes in the same family are mycorrhizal. The cultivar Lolita formed both vesicles and arbuscules when inoculated with either *G. mosseae* or *G. intraradices.* This cultivar was, however, not used in later experiments, as this genotype was withdrawn from production at Yoder Carnation, the supplier for cuttings. Since arbuscules are considered to be the site of nutrient exchange with the host (Hirrel et al., 1978), this indicates that a functional symbiosis was established between plant and VAM fungus. Except for a few vesicles, arbuscules were not observed in the association between Lavender Lace and *G. fasciculatum*. In cultivars Scania and Portrait, points of mycorrhizal infection as well as aseptate hyphae strongly resembling that of VAM were observed but no vesicles or arbuscules were observed. The roots did not appear to be infected with any other pathogen, probably because clean single-spore cultures of VAM were used as inoculum. With the lack of arbuscule formation in these three cultivars, it is questionable whether a functional symbiosis was established with the species of VAM fungi used in the study.

Whatever level of association there was between the VAM fungi and the carnation cultivars, it did affect the slopes of disease progression curves of carnation Fusarium wilt. The steeper disease progression curves in Lavender Lace with VAM fungi infection indicates that susceptibility to VAM of Lavender Lace is not necessarily a beneficial trait as it enhanced disease severity. Ross (1972) proposed that increase in disease severity due to VAM infection could be due to structures like chlamydospores and vesicles injuring plant tissue, creating avenues for penetration and development of pathogen. In this study, the VAM infection was so low that it could not be detected by the grid-line intersect method. The actual physical presence of VAM in roots is therefore unlikely to have been the cause of increased disease severity. There is the possibility of the VAM inducing physiological changes in the plant leading to increased disease (Modjo, 1983). This phenomena of VAM fungi infection enhancing disease severity should caution one against indiscriminate use of VAM fungi in commercial situations without extensive field testing. For mixed cropping systems such as is the case with carnation culture in Kenya, it also emphasizes the need to study a given VAM fungus and its hosts, not only in isolation, but also in the ecosystem they are to function in.

Disease severity levels were suppressed in both Portrait and Scania despite extremely low VAM infection levels, although the SNK test did not detect any significant differences in the maximum levels of disease attained by these cultivars with any of the VAM fungi treatments. However, this test for significance was not sensitive to the disease index ranks, and it equated a disease index of two with one of three (Table III-4 which is not valid. This may have been because the disease ranking data was not linear, and therefore this may not have been an appropriate method of analyzing such data. The slopes of the disease progression curves for the the two cultivars Scania and Portrait flattened with VAM infection. Although the slope flattening in Scania was minor (from 0.07 to 0.05), it may nevertheless be important from the point of view of integrated disease management. It may be possible to increase VAM infection levels in carnation roots by using a higher inoculum level than was used in this study. Infection level may not be the most important factor influencing the effect of VAM fungi on plants. Graham et al. (1982) found that plant growth improvement due to VAM infection is dependent on a minimum level of root colonization and degree of growth improvement is correlated with the extent of extraradical hyphae.

Many mechanisms have been suggested to explain how VAM fungi increase resistance of plants to root infecting pathogens. Morphological alterations of the host plant caused by mycorrhiza, such as thickening of cell walls through lignification and production of other polysaccharides, prevent growth and penetration of *Fusarium oxysporum* (Dehne and Schönbeck, 1979). Mycorrhizal fungi change their host plants both physiologically and biochemically (Ratnayake et al., 1976), effects which can be host genotype dependent. It was not surprising therefore that using the same VAM fungus species at similar inoculum levels, Fusarium wilt disease severity levels were suppressed more in one cultivar (Portrait) than in the other (Scania).

Parasitism of *G. intraradices* vesicles in Lolita roots may have led to the much lower final percent infection observed in *G. intraradices* infected roots of this cultivar, when compared with that attained using *G. mosseae*. This hyperparasitism is an important aspect in VAM research, as it could lead to variable results when different batches of the same mycorrhizal inoculum are used. Daniels and Menge (1980) reported that for plants such as citrus that are extremely dependent on myccorrhizal fungi for survival, hyperparasitism could greatly reduce levels of mycorrhizal infection leading to reductions in plant growth. In addition, roots of plants infected with *G. intraradices* were also infected with *Olpidium brassicae* and *Rhizoctonia* sp. These parasites could also have contributed to depression of level of infection by *G. intraradices*.

The data on plant dry weights and percent phosphorous content indicated that the *G. intraradices* infected plants had more dry matter than the uninoculated controls (except for Portrait.) However, inoculated plants had lower percentage phosphorous than their uninoculated counterparts at alpha = 0.05. This may have been due to the phosphorous in VAM inoculated plants being distributed over a bigger plant mass. Inoculation of carnation plants with VAM fungi therefore did not improve their phosphorous nutrition. Observed improved growth of the carnation is unlikely to have been due to improved phosphorous nutrition, but could be due to improved uptake of other nutrients. Uptake of nutrients such as K, Ca, Mg, Fe, Mn, Cu, Zn, Na, or B has been implicated in mycorrhizal assisted nutrition (Mosse, 1973). Production of growth promoting hormones by VAM fungi has also been implicated for improved plant growth of mycorrhizal plants (Krishna et al., 1981), but there has been no direct evidence of the transfer of these fungal hormones to the host plant in the symbiotic association.

Observations from the SEM study were consistent with what has already been reported (Baayen, 1987; Harling et al., 1984; Baayen et al., 1989; Pennypacker and Nelson, 1972). All these researchers reported plugging of vascular tissue in infected carnation stems. Pennypacker and Nelson (1972) tested for the contents of these plugs using differential stains and found that they contained pectins and wound gums. The plugs are thought to result from the lysis of primary cell walls and middle lamellae by pectolytic enzymes. They hypothesized that these enzymes split the constituents of the primary cell walls and middle lamellae into molecular fragments that then move into into the xylem vessel elements to form the amorphous plugs (Gothoskar et al., 1955;

Pierson et al., 1955). This study also found that these plugs sometimes contain calcium. The observed accumulations of calcium deposits that were mixed with gums could enhance the physical and chemical ability of the gums to block pathogen advancement. Calcium has been associated with induction of disease resistance in plants (Muchovej et al., 1980; Wills and Moore, 1967). Increase in calcium content in tomato sap has been shown to reduce Fusarium wilt incidence of tomato (Corden, 1965). In carnation, Blanc et al. (1983) reported that calcium deficiency at any stage of plant development increases Fusarium wilt. In the present study, it was observed that the resistant cultivar (Lavender Lace) showed more calcium deposition along cut surfaces of cell walls than did the other more susceptible cultivars. Baayen (1986) reported that hyperplasia in carnation is primarily a defence reaction associated with periderm formation and intended for localization of pathogens. Since calcium is a major cationic wall component of plant cells, this deposition could be indicative of actively dividing (hyperplastic) or enlarging (hypertrophy) cells, both of which indicate a resistance plant response. A good number of carnation resistance mechanisms to Fusarium wilt involve changes in the plant cells (Baayen, 1986). Thus, calcium may be a facilitator of resistance. Induction of disease resistance by calcium has also been attributed to the poor activity on calcium pectates by pathogens' pectinolytic enzymes. Several Fusarium wilt pathogens produce pectinases (Deese and Stahman., 1962; Edington et al., 1961; Heitefuss et al., 1960) which attack the cell walls and middle lamellae. This type of cell wall degeneration was noticed in this study more so in the susceptible Scania and

Portrait cultivars than in the more resistant Lavender Lace. This study therefore supported Blanc et al (1983). finding that calcium may reduce disease severity.

Studies of resistance to Fusarium wilt of carnation have to date focused mainly on physiological changes in the plant cells and some physical barriers localizing the pathogen (Baayen, 1988). The calcium component has to my knowledge not been much addressed. Studies elucidating the effect of VAM on carnation as well as action of calcium on Fusarium wilt of carnation may open up avenues of biological and cultural control of the disease, without necessarily compromising floricultural characteristics.

E. Tables, Figures, and Legends

Table III-1. ANOVA[†] for percent phosphorous content by weight and dry weights of three carnation cultivars[††] comparing VAM[§§] inoculated plants with uninoculated controls.

		MEAN SQUARE		F VALUE	
SOURCE	DF	Dry Wt	Phosphorus	Dry Wt	Phosphorus
CULT	2	2.114	0.002	1.020	3.27
VAM	1	42.483	0.008	20.570[§]	10.22[§]
CULT x VAM	2	5.101	0.0007	2.470	0.90
ERROR	24	2.066	0.0007		
TOTAL	29				

[§] Significant at alpha = 0.01.

[†] Analysis of variance.

[††] Portrait; Scania; Lavender lace.

[§§] *G. intraradices.*

Table III-2. Analysis of variance of plant dry weight[††] means[†] for three carnation cultivars.

VAM[§§]	ALL CULT. MEANS	SCANIA MEANS	L. LACE MEANS	PORTRAIT MEANS
+	7.787^a	7.680^a	8.160^a	7.520^a
–	5.407^b	6.500^a	4.200^b	5.520^a
Alpha	0.000	0.248	0.003	0.040[§]

[†] Means from five replicates. Means ending with the same letter are not significantly different by Student-Newman-Keuls (SNK) test at alpha = 0.01 within the same column.

[§] Significant at alpha = 0.05.

[††] Aerial part of plant only.

[§§] Two treatment levels: with VAM (+), without VAM (-).

Table III-3. Means separation for percent phosphorous content [††] of three carnation cultivars.

VAM[§§]	ALL CULT. MEANS[†].	SCANIA MEANS[†]	L. LACE MEANS[†]	PORTRAIT MEANS[†]
+	0.098[a]	0.104[a]	0.086[a]	0.104[a]
_	0.131[b]	0.126[a]	0.110[a]	0.156[a]
Alpha	0.003	0.234	0.082	0.050[§]

[†] Means from five replicates. Means ending with the same letter are not significantly different by Student-Newman-Keuls (SNK) test at alpha = 0.01 within the same column.

[§] Significant at alpha = 0.05.

[††] Aerial part of plant only.

[§§] Two treatment levels: with VAM (+), without VAM (-).

Table III-4. Analysis of variance of disease indices at K_{max}*for three cultivars of carnation inoculated with four treatments of vescicular-arbuscular-mycorrhizal fungi.

CULT	DISEASE INDEX MEANS[1]			
	NO VAM	*G. fasciculatum*	*G. mosseae*	*G. intraradices*
SCANIA	3.60^a	2.90^a	3.40^a	3.50^a
PORTRAIT	3.20^a	2.30^a	3.00^a	3.10^a
L. LACE	1.80^b	2.30^a	2.00^a	2.00^a

*K_{max} = maximum level of disease attained during the disease epidemic.

[1] Means from five replicates. Means ending with the same letter are not significantly different by Student-Newman-Keuls (SNK) test at alpha = 0.01 within the same column.

Table III-5. Summary of slope (B) and coeffecients determination (R^2) values of disease progress curves[†] for three carnation cultivars and four VAM treatments.

TREATMENT	CULTIVARS					
	SCANIA		PORTRAIT		L. LACE	
	B	R^2	B	R^2	B	R^2
NO VAM[††]	0.07	0.99	0.06	0.99	0.02	0.99
G. fasciculatum	0.07	0.99	0.03	0.98	0.05*	0.99
G. mosseae	0.05	0.99	0.05	0.99	0.03*	0.98
G. intraradices	0.05	0.97	0.04	0.98	0.03*	0.98

[†] as determined using the Gompartz model.

[††] control.

*slope steeper than control.

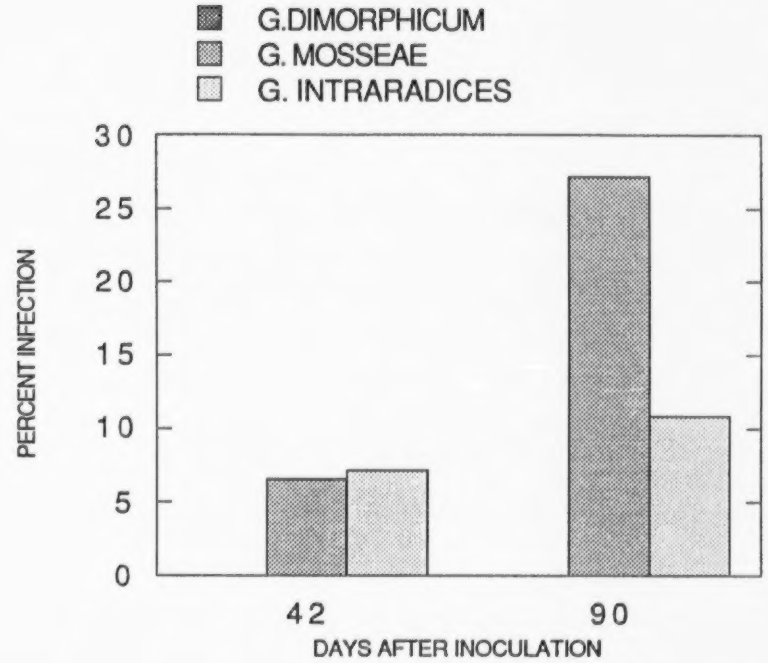


Fig. III-0. Percent infection† of carnation cultivar Lolita roots with vescicular-arbuscular mycorrhizal fungi.
†only vesicular infection was scored.

Fig. III-1. (× 500) Carnation roots infected with *Glomus intraradices* showing contamination by:

- X *Olpidium brassicae*. Note the ornamental resting spores
- Y *Rhizoctonia* sp. microsclerotia
- Z a *Lagena radicicola*-like organism. Note thick-walled spores

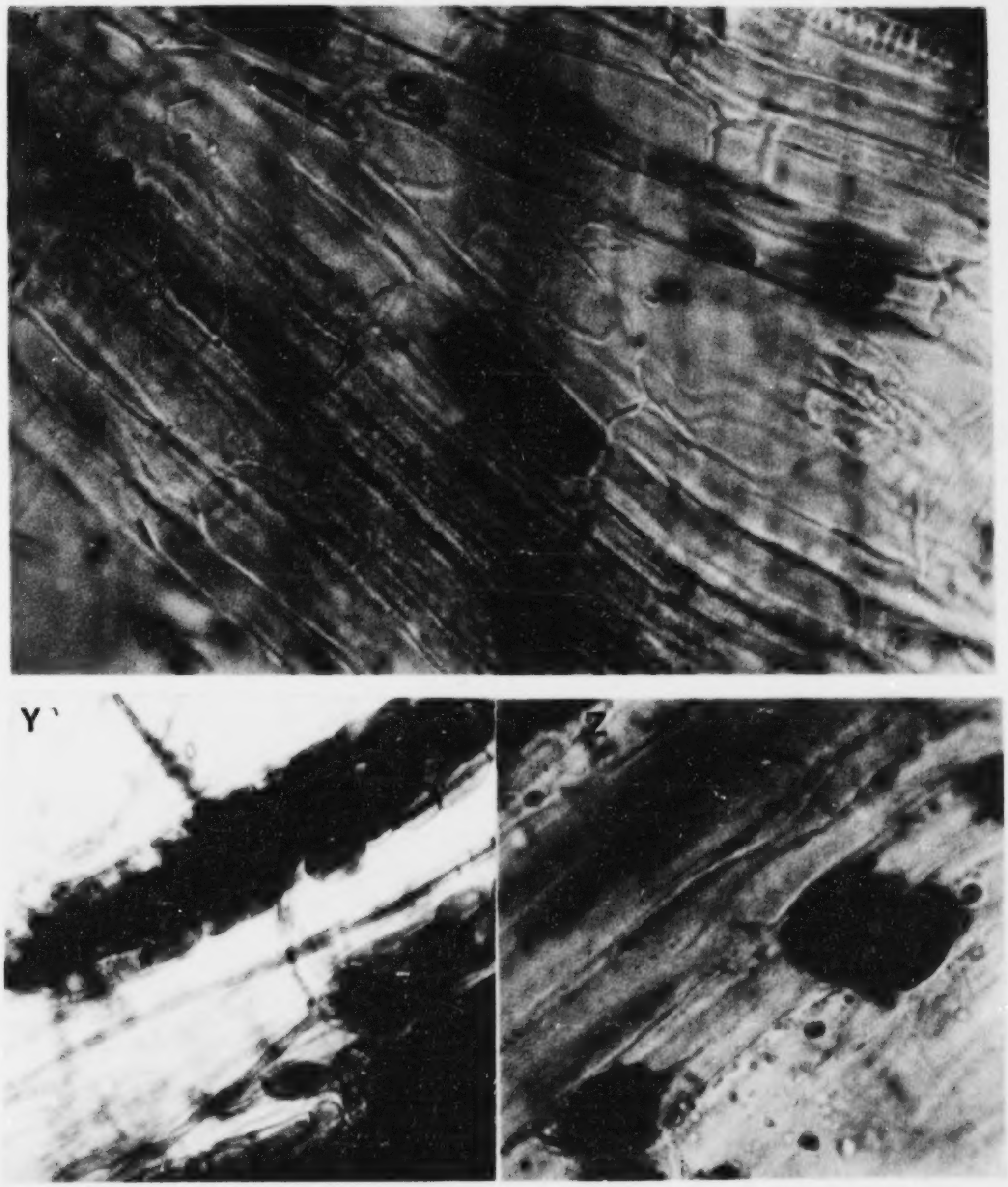

Y
Z

Fig. III-2. (× 500) *Glomus intraradices* vesicles in carnation root parasitized by a *Lagena radicicola*-like organism

X Note the lysis of a vesicle releasing thick-walled spores (arrow)

Y Note the organism infects both root (arrowhead) and vesicle (arrow)

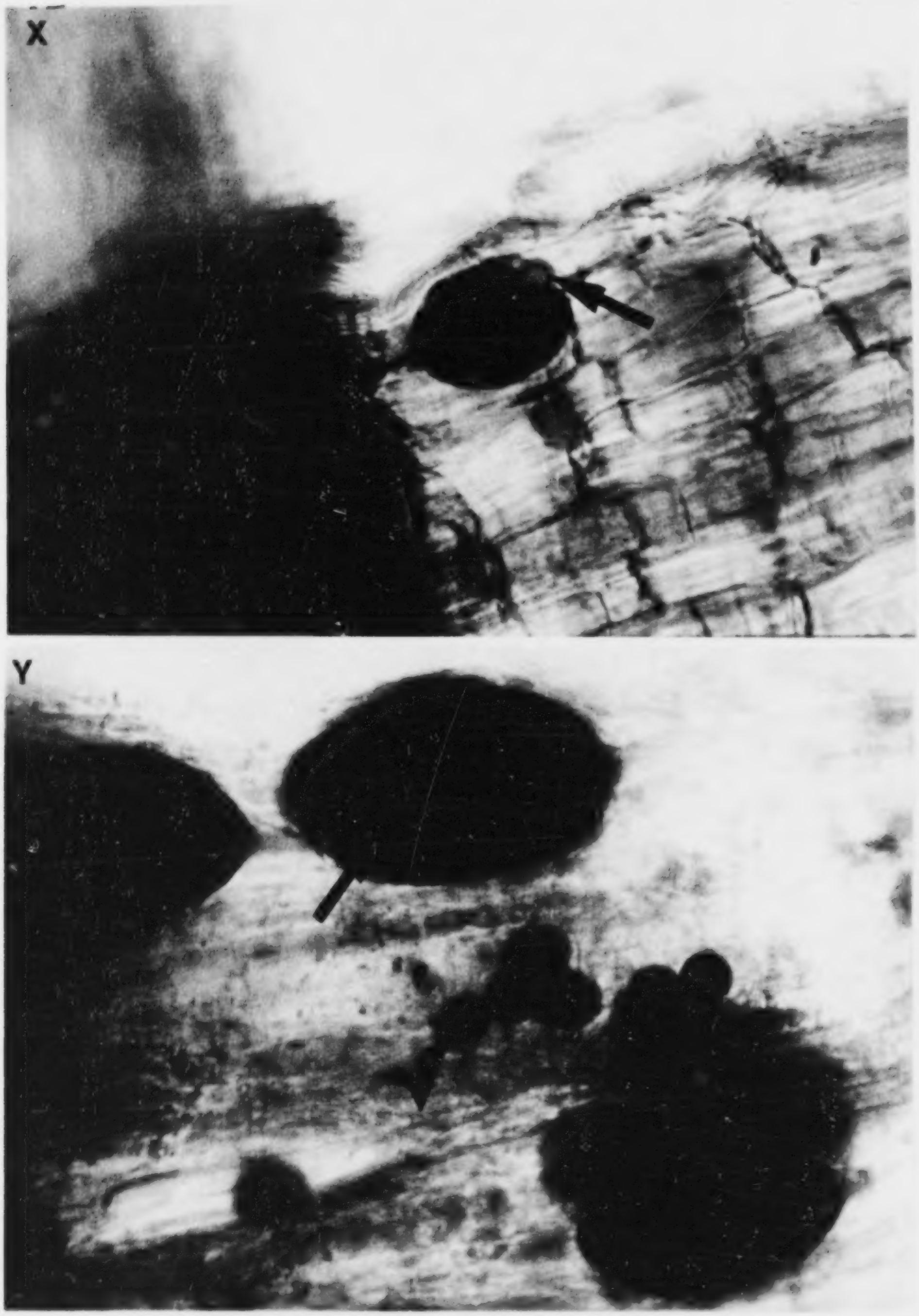
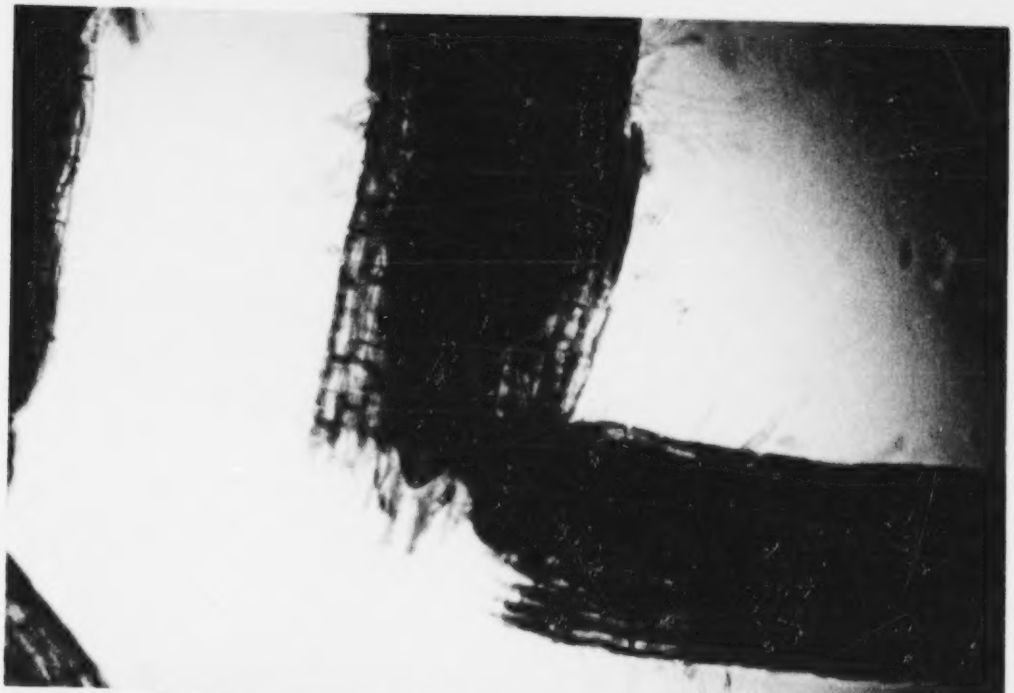

X
Y

Fig. III-3. (× 500) Vesicular infection of carnation cv. Lavender Lace with *G. fasciculatum*



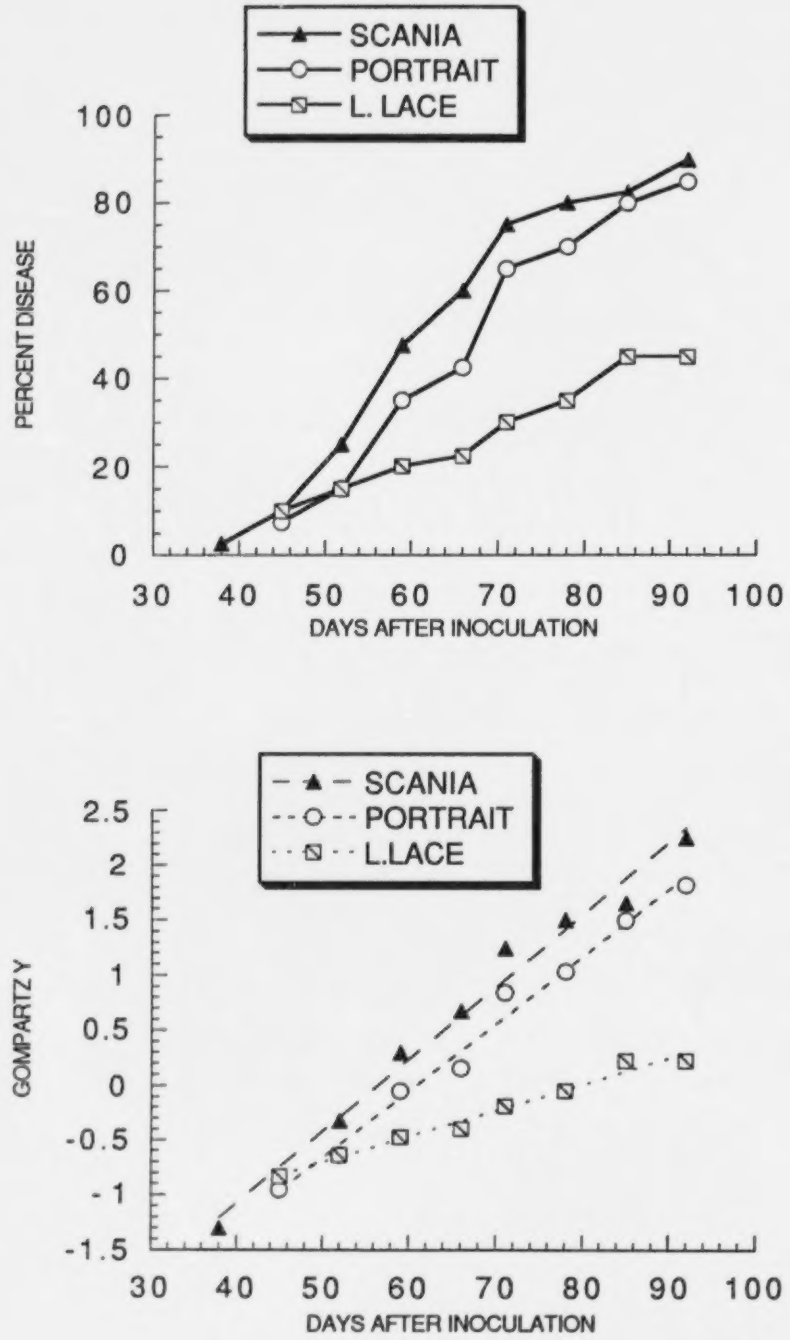


Fig. III-4a. Actual and predicted Fusarium wilt disease severity values plotted against time for carnation cultivars Portrait, Scania, and Lavender Lace, and *Fusarium oxysporum* f. sp. *dianthi*.

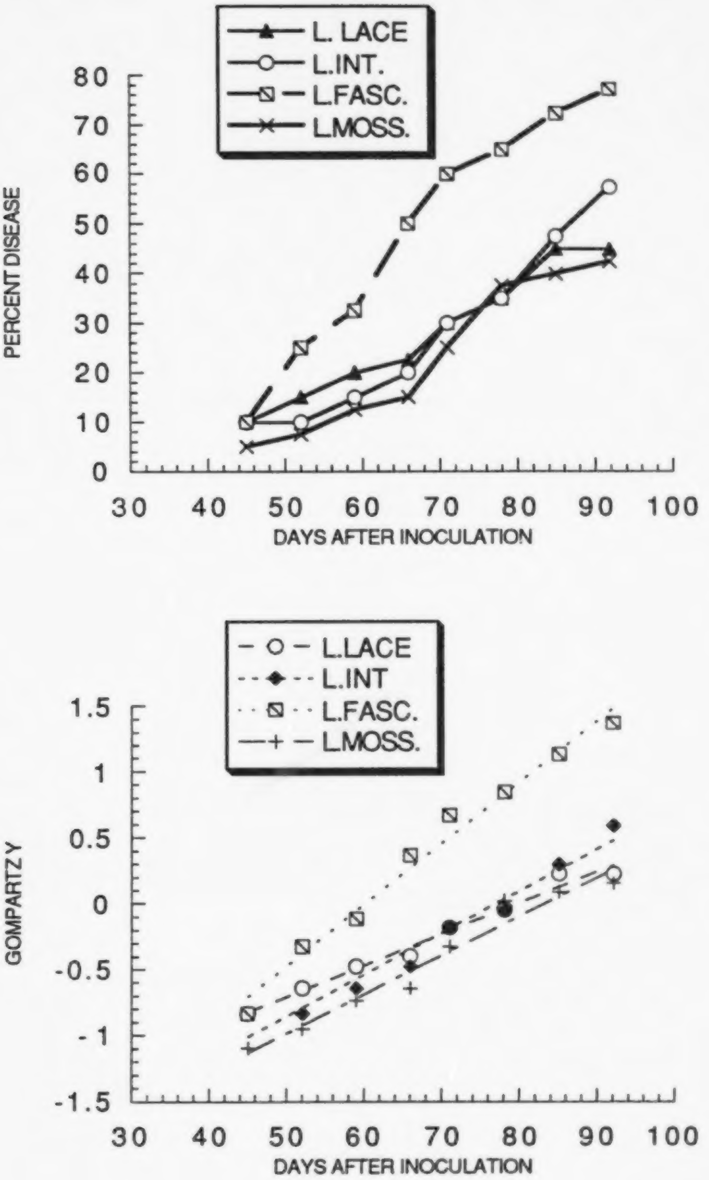


Fig. III-4b. Actual and predicted Fusarium wilt disease severity values plotted against time for carnation cultivar Lavender Lace, inoculated with *Fusarium oxysporum* f. sp. *dianthi*.† and three VAM spp: *Glomus fasciculatum*, *G. intraradices*, and *G. mosseae*.
†Strain B of *F. o.* f. sp. *dianthi* was used.

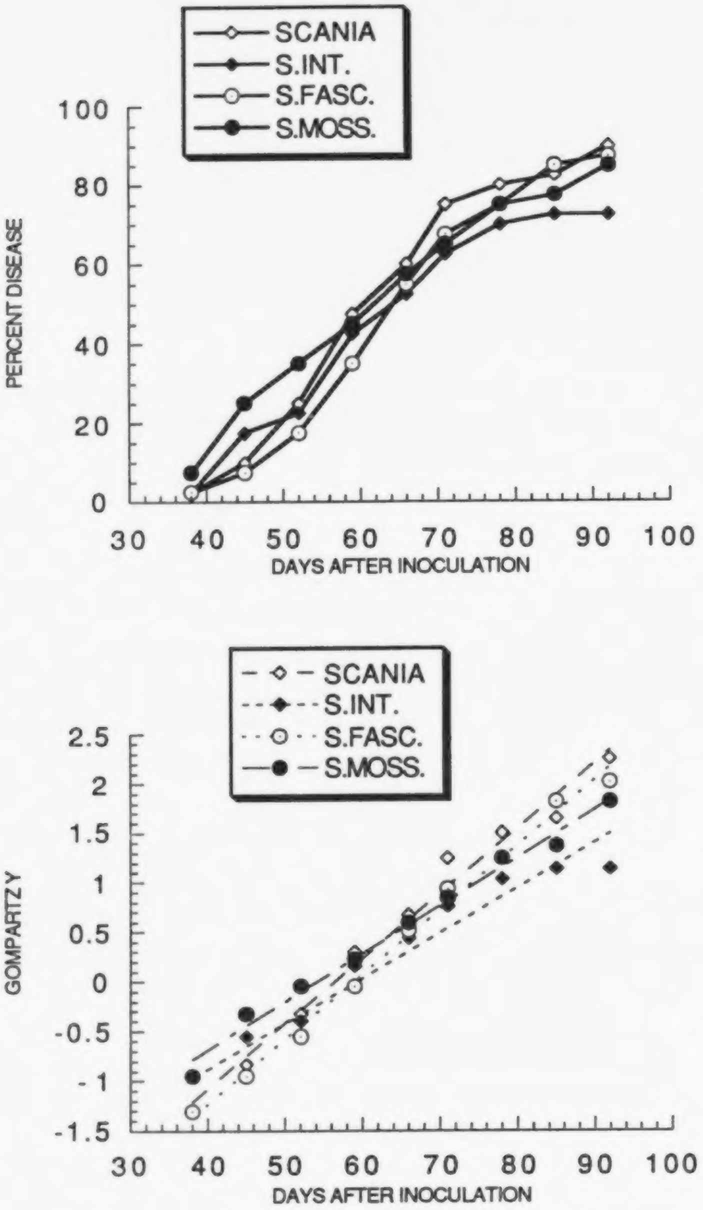


Fig. III-4c. Actual and predicted Fusarium wilt disease severity values plotted against time for carnation cultivar Scania, inoculated with *Fusarium oxysporum* f. sp. *dianthi*.[†] and three VAM spp: *Glomus fasciculatum*, *G. intraradices*, and *G. mosseae*.

[†] Strain B of *F. o.* f. sp. *dianthi* was used.

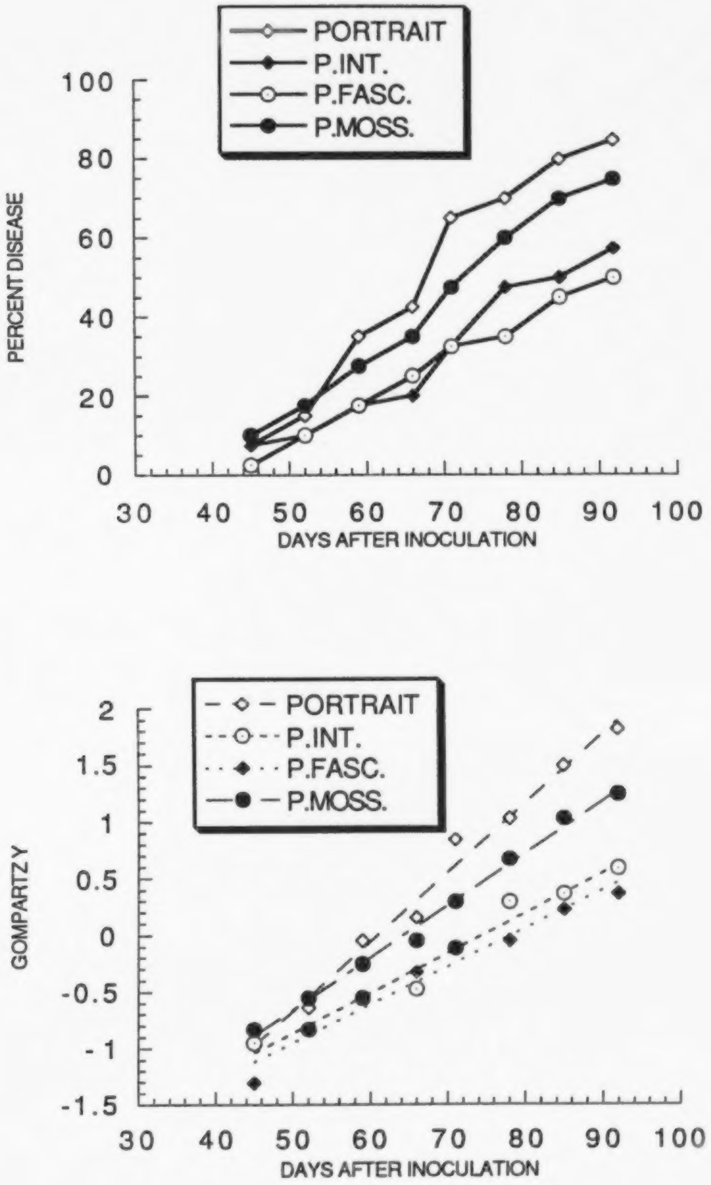


Fig. III-4d. Actual and predicted Fusarium wilt disease severity values plotted against time for carnation cultivar Portrait, inoculated with *Fusarium oxysporum* f. sp. *dianthi*.† and three VAM spp: *Glomus fasciculatum*, *G. intraradices*, and *G. mosseae*.
†Strain B of *F. o.* f. sp. *dianthi* was used.

Fig. III-5. A scanning electron micrograph of a cross section of a disease-free carnation stem showing:

Epidermis (E), Fiber cylinder (F), cortex (C), vascular tissue (V), and pith (P)

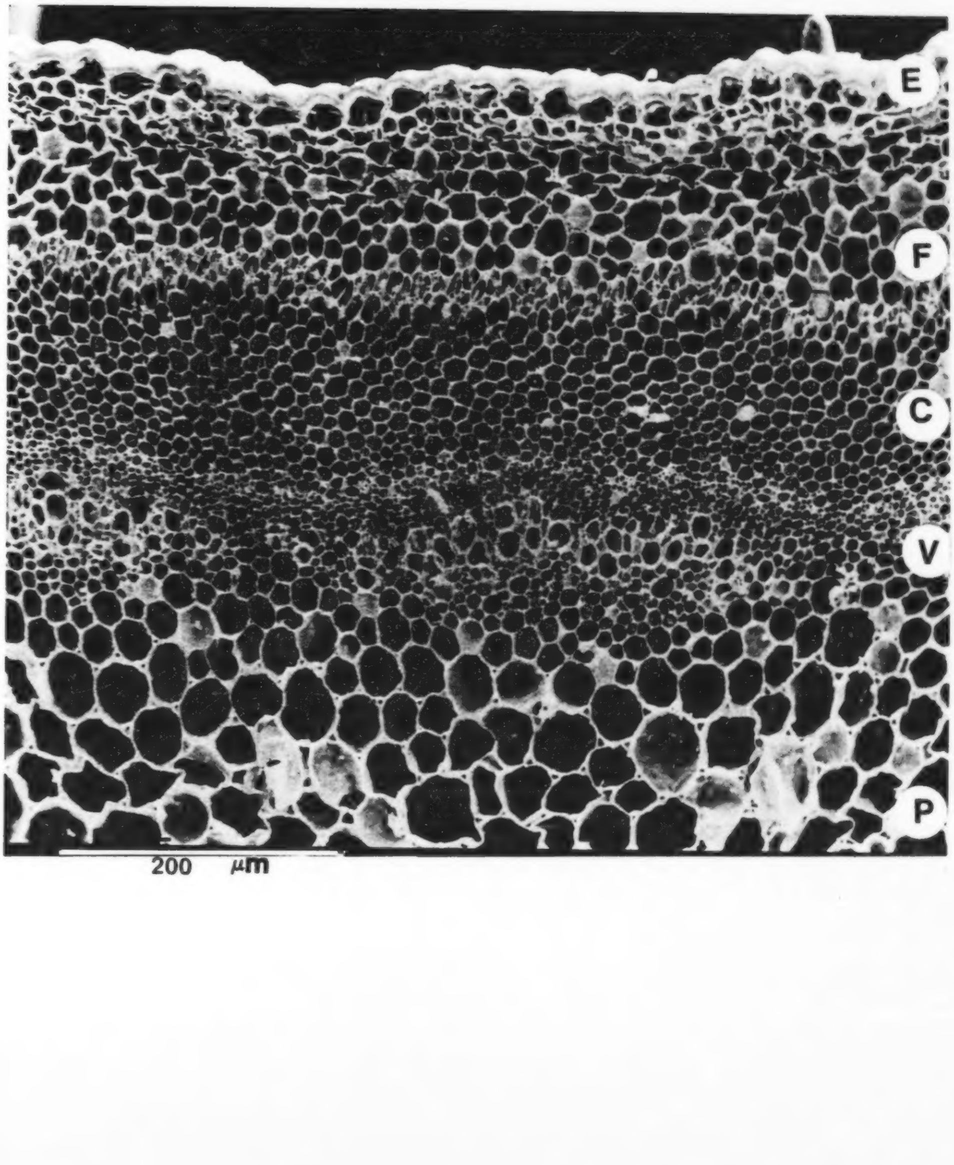

E
F
C
V
P
200 μm

Fig III-6. Scanning electron micrographs showing parts of cross and long sections from stems of cv. Portrait infected with *F. o. dianthi*

X cross section from mid way up the stem of cv. Portrait showing intercellular as well as intracellular colonization of xylem tissue

Y long section showing vertical spread of the fungus lateral

Z a cross section showing lateral spread of the fungus into the cortex. Note differentiation of cortex cells into pitted xylem (arrowhead)

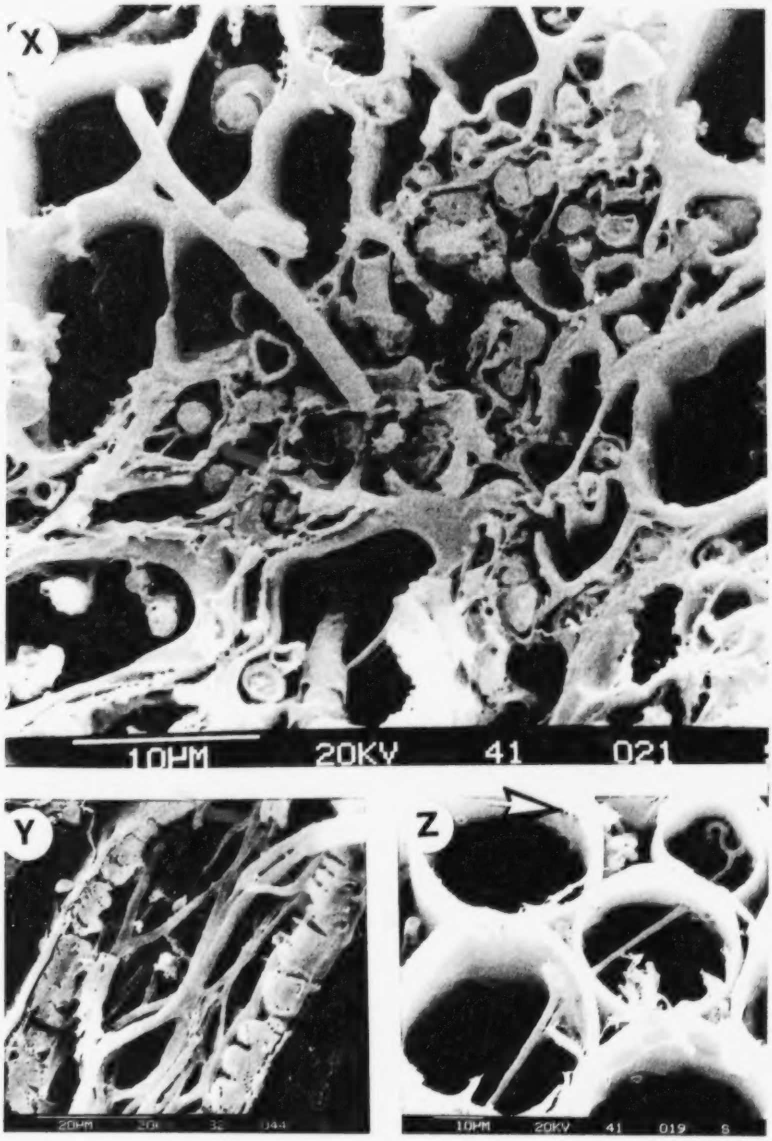

X
10µM 20KV 41 021
Y
20µM 20K 32 044
Z
10µM 20KV 41 019 S

Fig III-7. Scanning electron micrographs of parts of long sections taken from *F. o. dianthi* infected Scania stems showing disitegration of infected plant cells

X Note lateral spread of fungus (arrow)

Y Note extensive disintegration leading to formation of cavities (arrows)

Z Note chlamydospores (arrow)

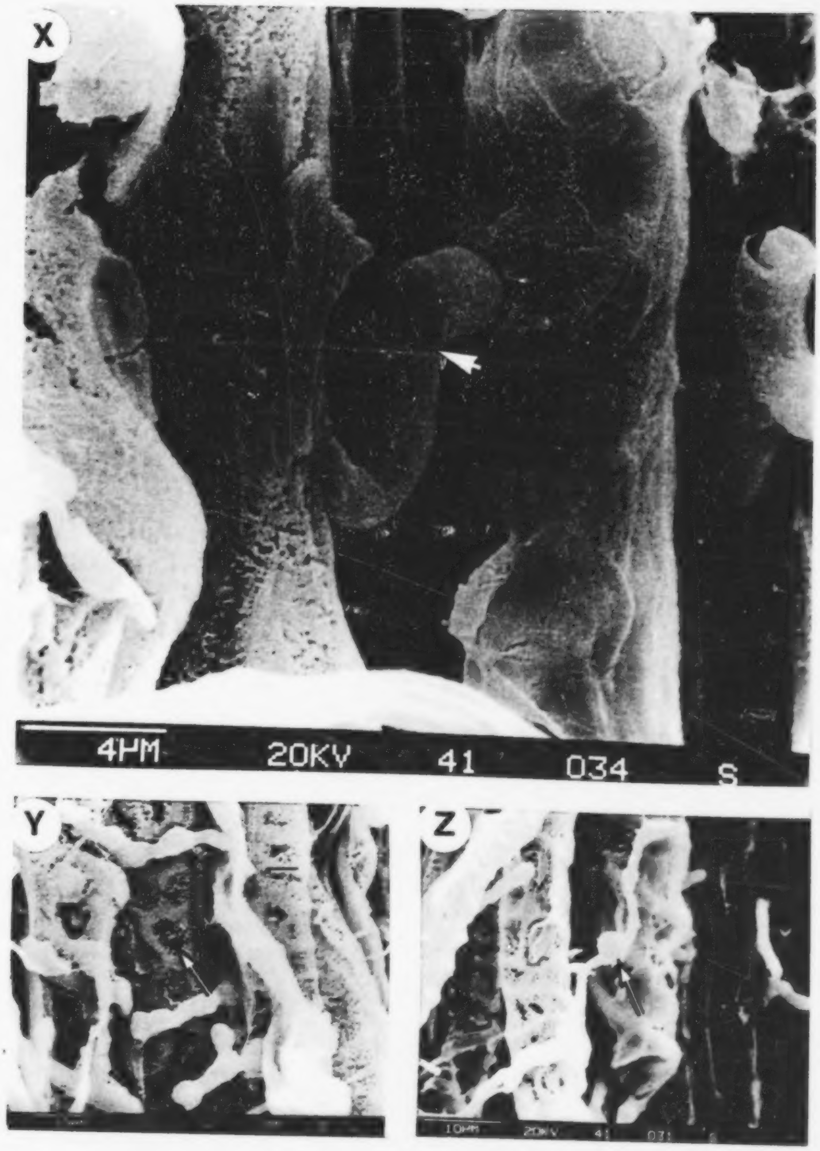
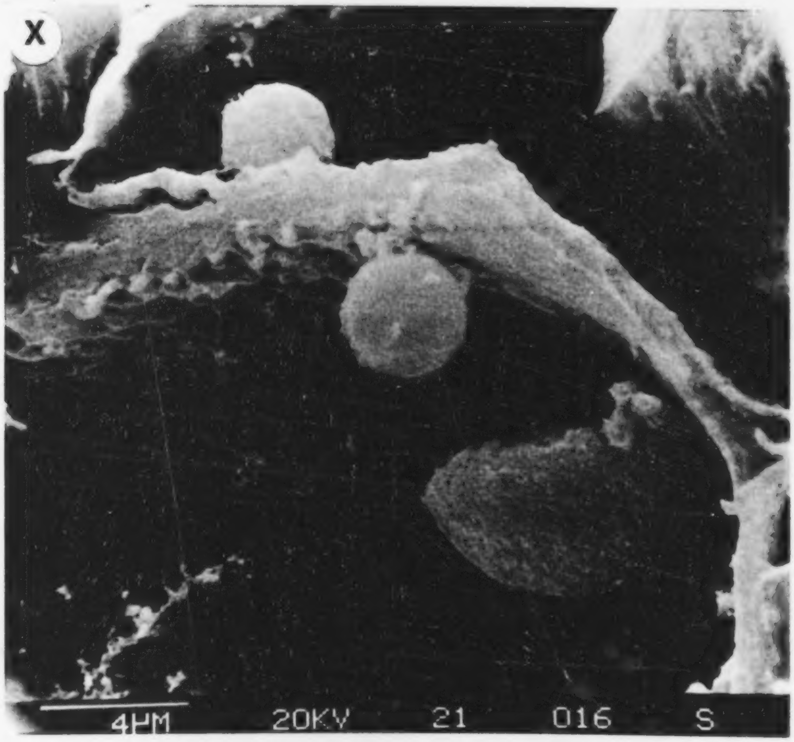

X
4HM 20KV 41 034 S
Y
Z
10HM 20KV 41 031 S

Fig III-8. Scanning electron micrographs of parts of long sections from *F. o. dianthi* infected Portrait and Lavender Lace stems, showing 'drop' gum deposits at xylem pits

X Portrait basal part of the stem

Y Lavender lace basal part of the stem


X
4µM
20KV
21
016
S

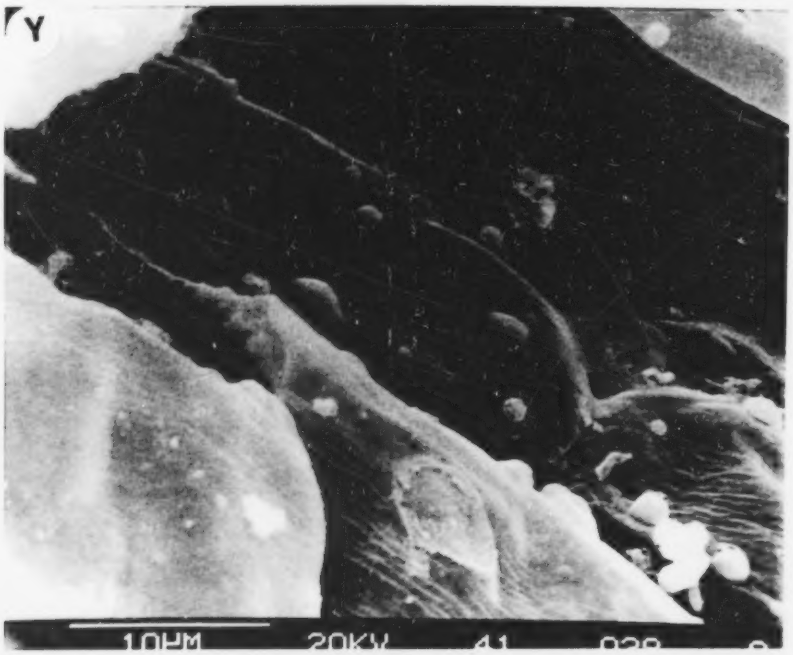
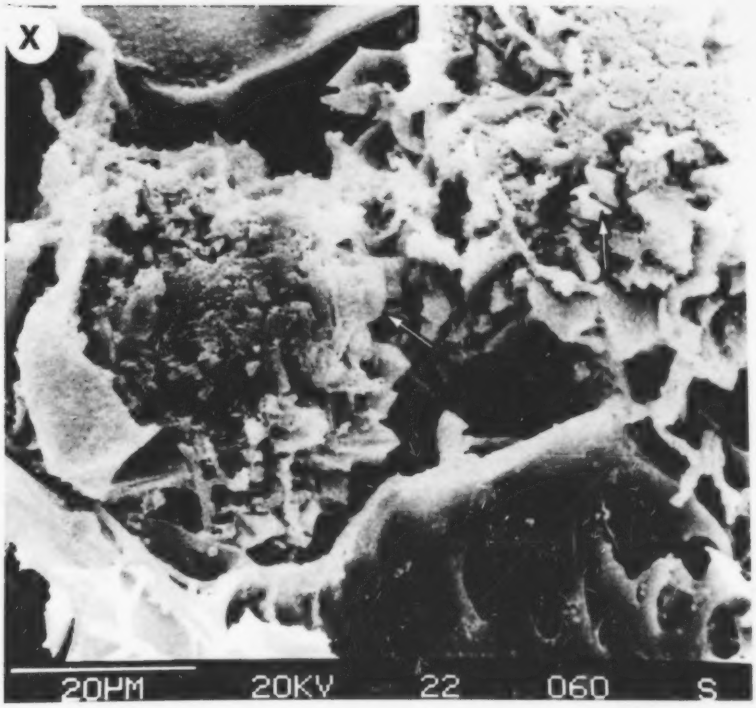

Y
10µM
20KV
41

Fig III-9. Scanning electron micrographs of cross sections of *F. o. dianthi* infected carnation stems, showing occlusion of the vascular tissue

X Section from the mid-stem of Portrait. Note mixture of calcium crystals with amorphous gums

Y Section from the tip of a Scania stem. Occlusion by amorphous gels. Note thickened walls (arrows)


X
20PM 20KV 22 060 S

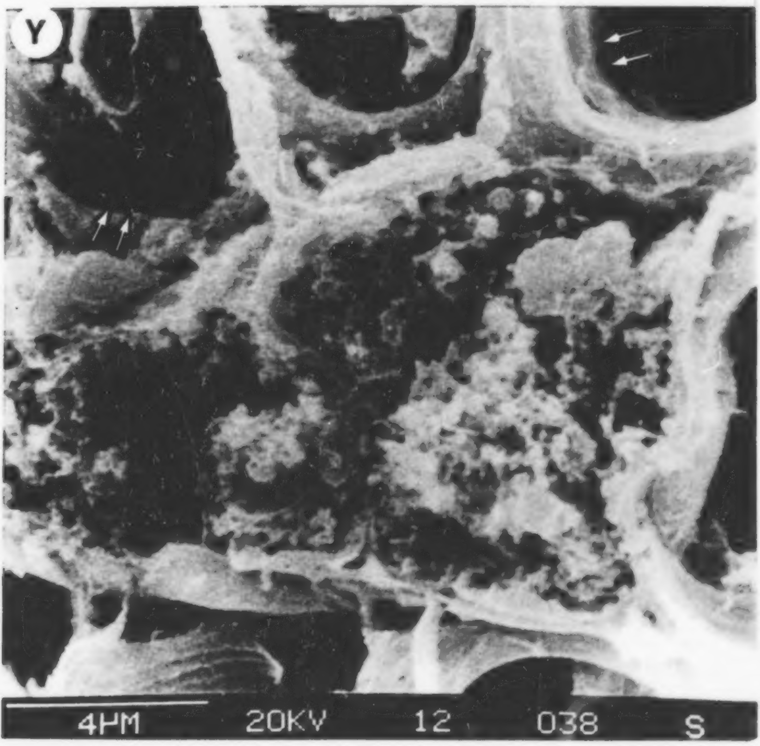
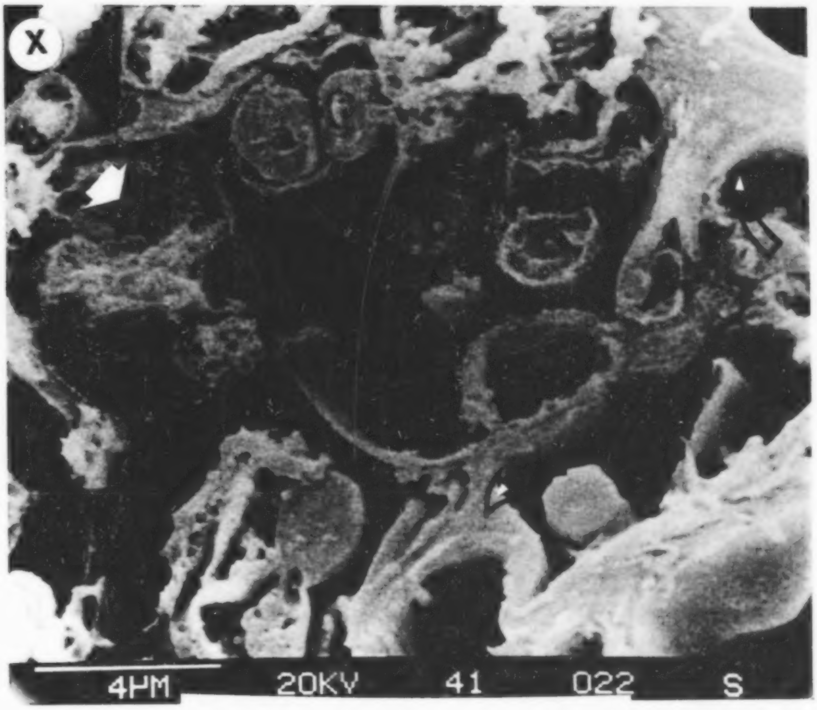

Y
4PM 20KV 12 038 S

Fig. III-10. Scanning electron micrographs of parts of cross sections from *F. o. dianthi* infected stems of Scania showing:

X cell wall disintegration (arrowhead). Note the thickened cell walls of cells adjacent to infected xylem (hollow arrows)

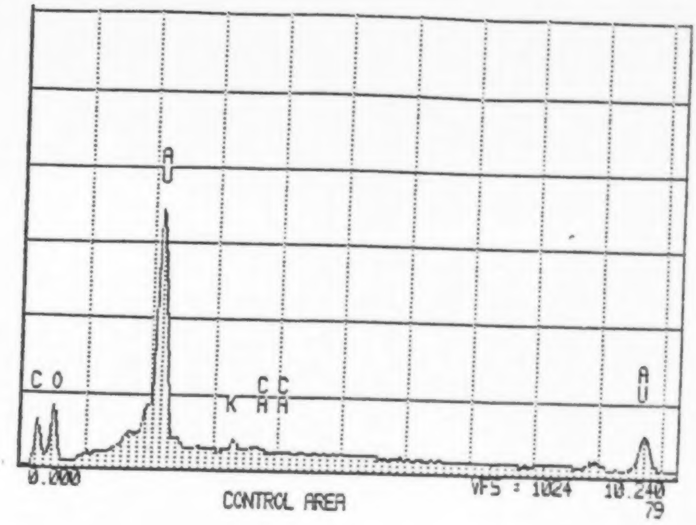
Y Thickened walls.of parenchyma cells next to infected xylem vessels at higher magnification


X
4µM 20KV 41 022 S

Y

Fig. III-11. Scanning electron microscope x-ray spectra comparing an area in a *F. o. dianthi* infected stem of a Scania plant that had calcium deposition and one without

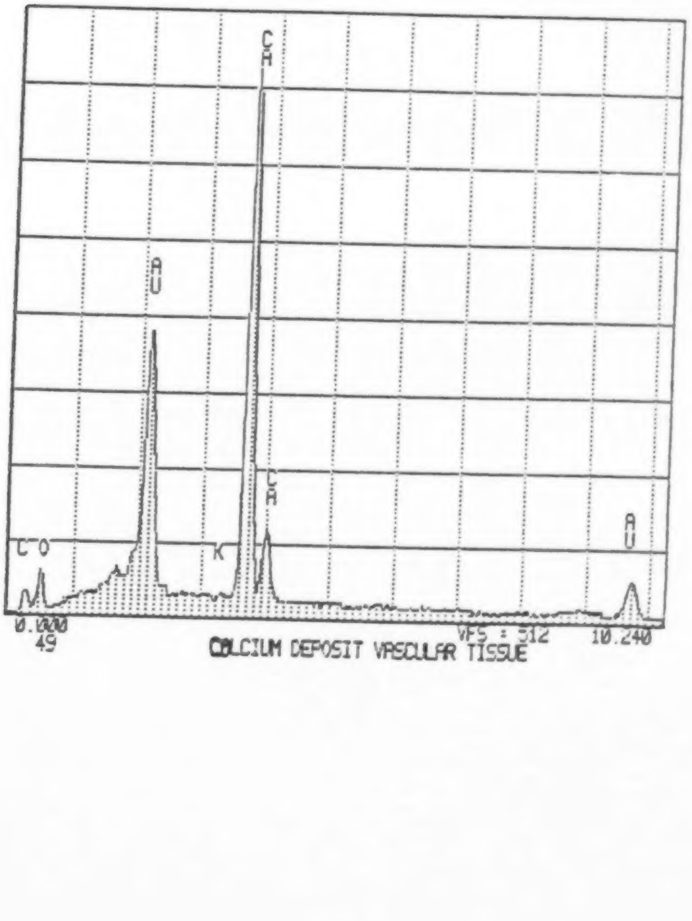
Note the calcium peak in the spectrum for the calcium deposition area



CONTROL AREA

ENTOMOLOGY SEM FACILITY
Cursor: 0.000KeV = 0

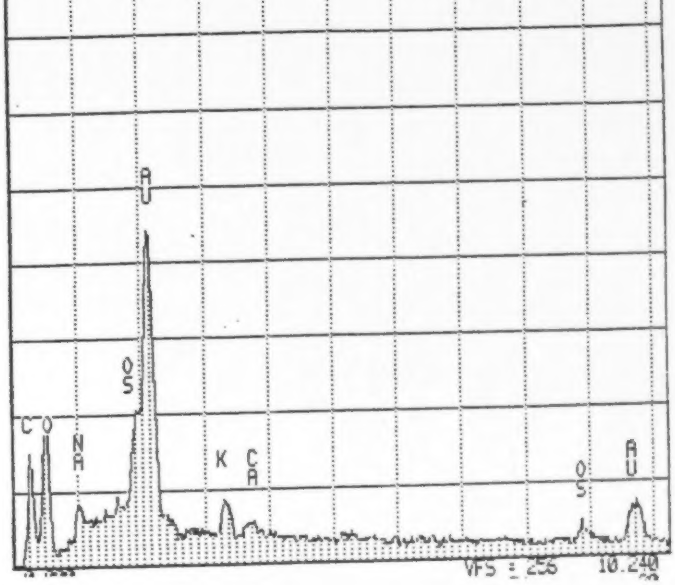
MON 20-JUL-92 10:03



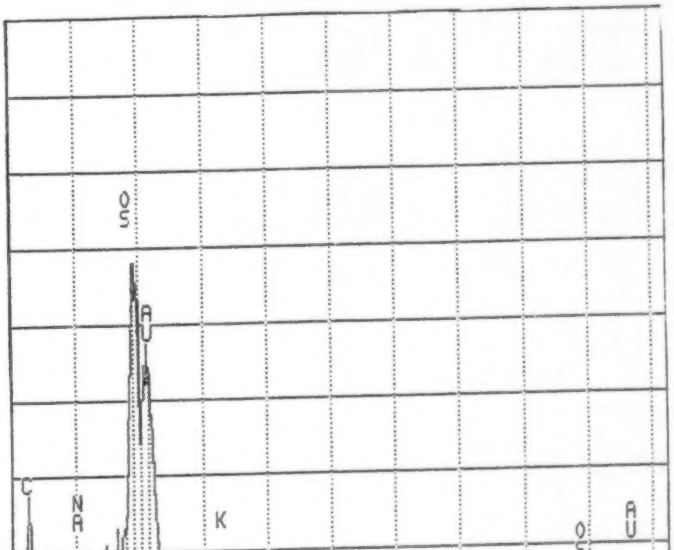
CALCIUM DEPOSIT VASCULAR TISSUE

Fig. III-12. Scanning electron microscope x-ray spectra of a section of a *F. o. dianthi* infected carnation plant, comparing an area with gum deposition and one without

Note the high lipid content of gum as suggested by the osmium peak


AU
OS
C
O
NA
K
CA
OS
AU
VFS = 256
10.240

control.


OS
AU
C
NA
O
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OS
AU
0.000
30
SAMPLE 5B GUMMY SUBSTANCE
VFS = 256
10.240

Fig III-13. Scanning electron micrographs of parts of cross section cuts of *F. o. dianthi* infected mid-stems of Lavender Lace, showing calcium crystal deposition along the cut surfaces of cell walls

X.. Note thickened walls (arrowheads) and lack of fungal hyphae

Y A higher magnification of Fig. 4.7 X

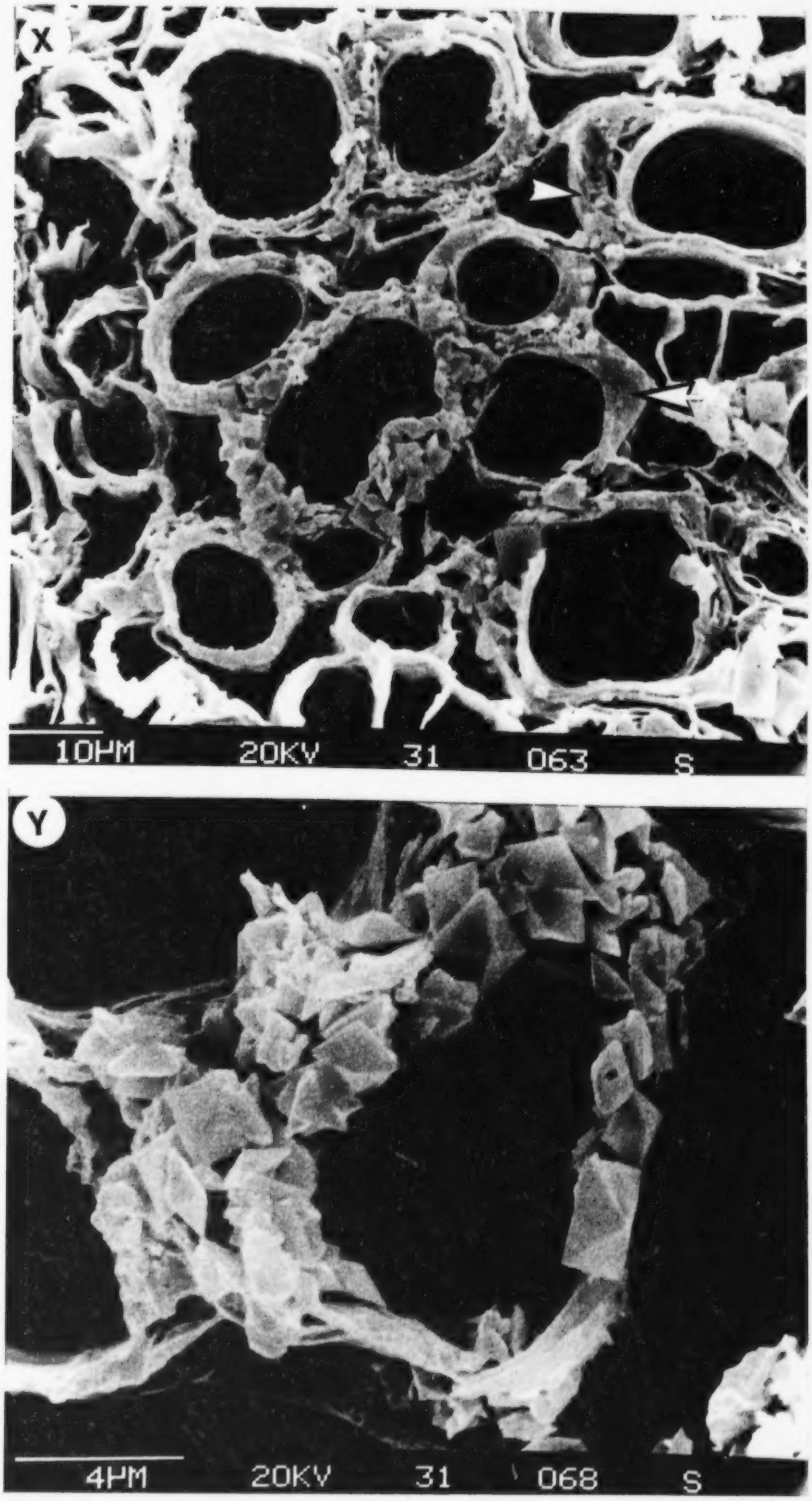

X
10µM 20KV 31 063 S
Y
4µM 20KV 31 068 S

Fig. III-14. Scanning electrone microscope x-ray spectrum showing high levels of calcium along cell walls of *F. o. dianthi* infected carnation plants

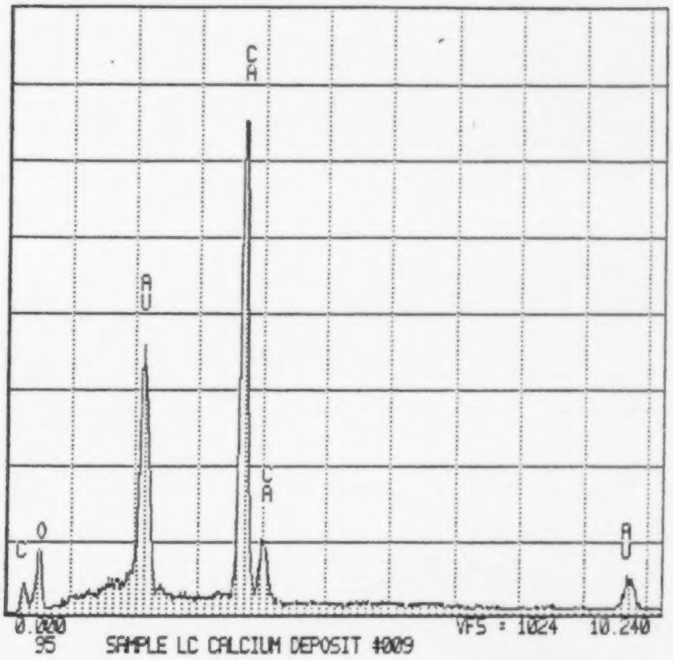
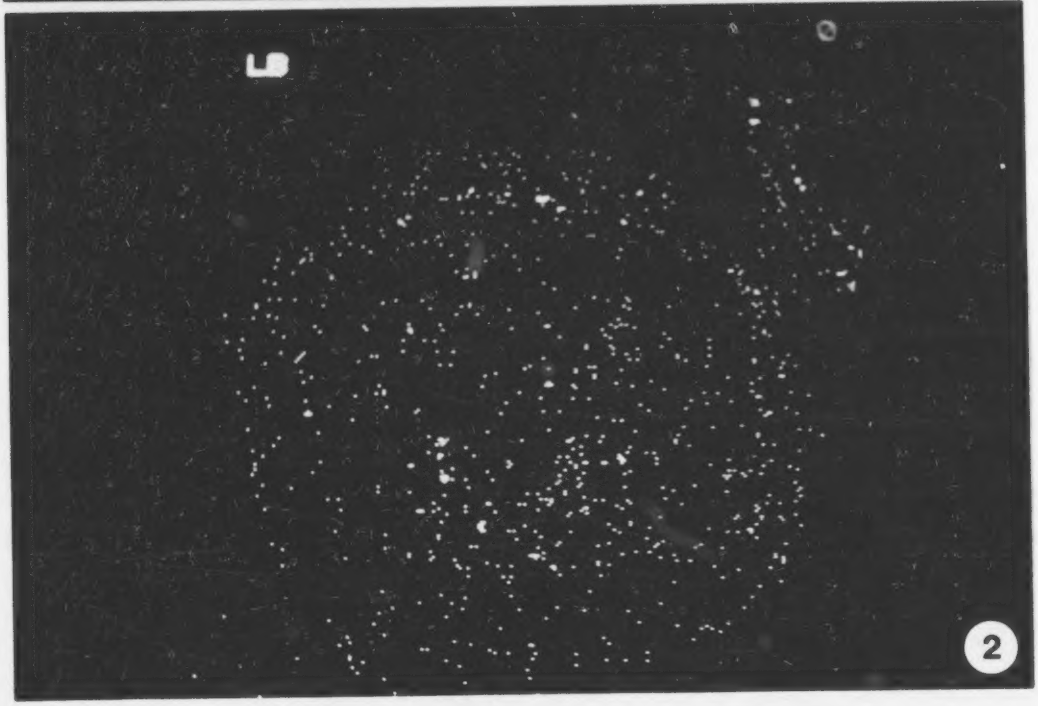

CA
AU
CA
C
O
AU
0.000
95
SAMPLE LC CALCIUM DEPOSIT #009
VFS = 1024
10.240

Fig. III-15. Scanning electron microscope x-ray mapping of calcium comparing suceptible cv. Scania (A) and the resistant cv. L. Lace (.B) 1 = control; 2 = infected

A
1

SB
2

1



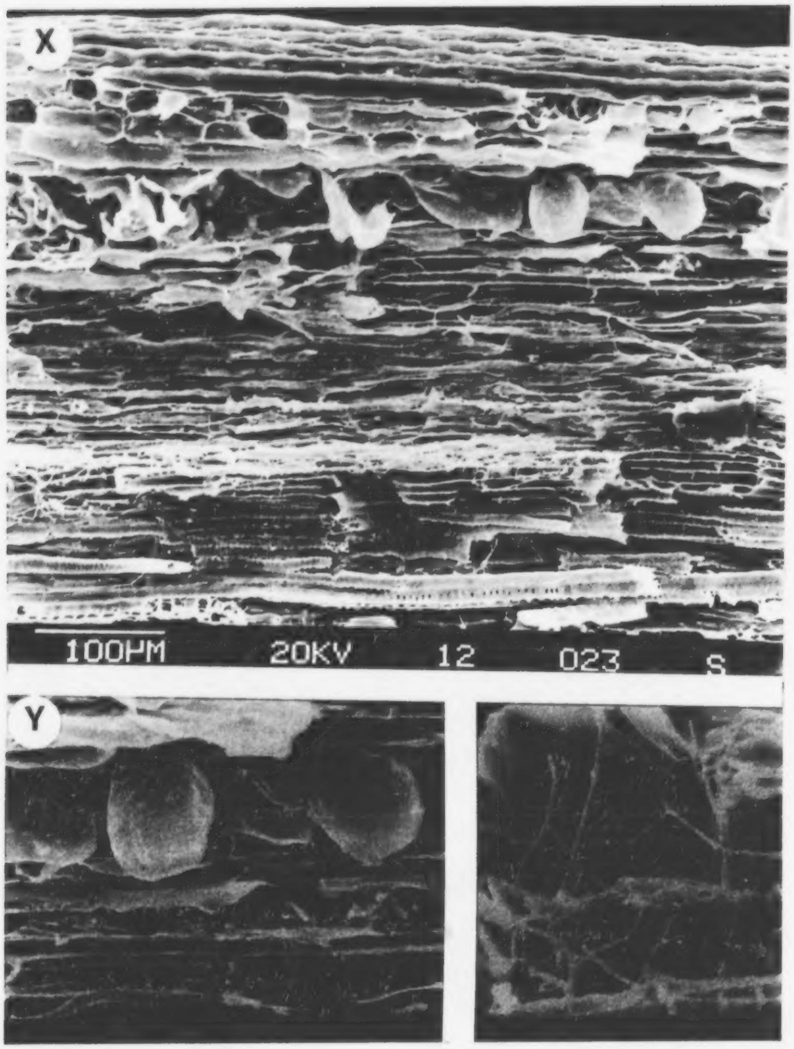
2

Fig. III-16 Cross section of the mid stem of *F. o. dianthi* infected Scania plants

X Note the tyloses creating a physical barrier in the xylem.

Y X at a higher magnification

Z Note fungal hyphae trying to get past the vesicles


X
100PM
20KV
12
023
S
Y

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Chapter IV

Cryopreservation of VAM Fungi and Taxonomy of VAM Fungi From Carnation Growing Areas in Kenya

A. Introduction

The benefits of VAM fungi are greatest and most obvious under low input growing conditions such as are found in most tropical developing countries (Nicolson and Johnston, 1974). The VAM fungi are also known to improve soil structure through increased soil aggregation, an important factor when considering erosion prone tropical soils. Colonization by VAM fungi is also known to reduce severity of plant diseases, especially soil borne pathogens (Bali et al., 1987) by physical as well as physiological alteration of the host plant (Dehne, 1982). The scope for exploiting these fungi to increase yields of economically important plants and to insure continued productivity of forestry and agriculture in the tropics is therefore very high, and it is therefore imperative to catalog, understand, and preserve tropical VAM fungi (Janos, 1987).

A mycorrhiza survey carried out in the semi-arid sahel region of Senegal reported presence of species of three VAM fungi genera in the area studied; *Glomus*, *Gigaspora*, and *Sclerocystis* (Diem et al., 1981). Sward et al. (1978) showed the Cranbourne heath in Australia to be rich in non-sporocarpic members of the Endogonaceae. They also described eight different spore types, spanning the genera *Glomus*, *Gigaspora*, and *Acaulospora*. Redhead, (1977) recorded the occurrence of several Endogonaceae species that had been previously recorded in U.S.A., Scotland, and Australia in Nigeria. He also reported several species that had not been previously described.

Microbes have an inherent genetic instability (Kidby, 1977), hence development of genotype preservation techniques deserves high priority. Although no method ensures complete preservation, substantial preservation may be achieved by freezing, L-drying, and lyophilizing. (Annear, 1956; Kidby, 1977; Lampage et al., 1970). Tommerup and Kidby (1979) reported successful storage and preservation of VAM fungal spores by L-drying. Another possible technique that has not been investigated for VAM fungal spore storage is cryopreservation in liquid nitrogen. Liquid nitrogen has been used to successfully preserve cultures, tissues, and spores (Hwang, 1966; Smith, 1982).

Rapid viability tests are very useful in setting up schemes for long-term preservation (Tommerup, 1983). Germination is used in many instances as a measure of viability, but results are complicated by spore dormancy (Tommerup, 1983), and research on dormancy has been hampered by a lack of independent measures of viability (An and Hendrix, 1988). Use of vital stains to determine viability of endogonaceous spores has been minimal. Daniels Hetrick (as cited by Menge and Timmer, 1982) tested triphenyl tetrazolium chloride and rose bengal and found it difficult to differentiate living from dead spores. One percent aqueous thionin differentiates between living and dead spores, but its use as a vital stain is limited, as experience is required for it to be effectively used (Menge and Timmer, 1982).

An and Hendrix (1988) used 3-(4,5-dimethylthiazol-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to successfully determine viability of two *Glomus* spp.; *G. macrocarpum* Tul. & Tul. and *G. mosseae* (Nicol. & Gerd.) Gerdemann & Trappe. They tested the validity of the vital stain procedure with a *G. mosseae* culture because it does not exhibit spore dormancy. They found that MTT stained living spores a bright red. Spores killed by autoclaving or by ethanol retained their normal unstained appearance at 40 hours, after which they turned blue, while spores that had apparently died during storage turned blue before 40 hours had elapsed.

The MTT vital stain had previously been used to determine viability of *Phytophthora* spp. oospores (Cohen, 1984; Sutherland and Cohen, 1983). The chemistry of tetrazolium salts is reviewed by Altman (1976). The salt is reduced by cellular dehydrogenase(s) to form a colored formazan product. This deposit is the most important characteristic of tetrazolium salts from a histochemical point of view. The MTT formazan deposit is crystalline; purple, blue or grey in color; becoming pink when dissolved in lipid (Altman, 1976). Another vital stain [indonitro tetrazolium (INT)] has been found useful in quantifying active extraradical hyphae of VAM fungi, allowing for the assessment of VAM fungal growth in soil (Sylvia, 1988).

The current study was undertaken to identify some VAM endophytes indigenous to some carnation growing areas of Kenya. Cryopreservation of VAM fungi spores was also investigated as an option for long time preservation of spores. The success of using MTT as a vital stain was investigated using four *Glomus* spp: *G. fasciculatum* (Thaxter) Gerd. & Trappe amend. Walker & Koske, *G.*

intraradices Schenck & Smith, *G. dimorphicum* Boyetchko & Tewari, and *G. mosseae.*

B. Materials and Methods

1. Soil Sampling

Soil samples were collected from carnation growing fields of Kiambu and Naivasha in Kenya in June-July 1990 and 1991. The samples were taken from the top 30 cm of soil. From Naivasha, soil sampling was done on one farm only. From Kiambu, samples were taken from three spatially separated carnation growing localities; Kikuyu, Limuru, and Githunguri. Three farms from each locality were sampled. These soils are described as well drained, shallow, dark-reddish brown, friable, calcareous, bouldery, ferric to clay loams, and are usually saline (Farm Management Handbook, Ministry of Agriculture, Kenya, 1979). The soils from Naivasha had 7.9% extractable phosphorous and those from Kiambu had 15.3% (Agri-Service laboratory Inc. Kitchener, Ontario). The soil samples were packed in polythene bags sealed and brought to Canada where they were stored at 4 C.

Spores were isolated from each of the soils and were mounted in water, polyvinyl-lactic acid (PVLA) and Melzers reagent:PVLA 1:1 (MPVLA). The PVLA was prepared from 1.66 g polyvinyl alcohol, 10 ml distilled water, 10 ml lactic acid, and 10 ml glycerine (Salmon, 1954).

2. Isolation of VAM Fungi Spores

Soil (100 g) was mixed with 200 ml of water in a waring blender, and blended intermittently for 30 seconds on a low speed to dislodge spores. The mixture was then decanted successively onto two sieves, with pore sizes 425 and 45 mm, and tap water allowed to wash through them for about a minute. The residue on the 45 mm sieve was washed into a beaker. The spores in this washing were rescued using a modification of the sucrose gradient centrifugation (Ianson and Allen, 1986). Ten ml aliquot of 60% sucrose solution as pipetted below a 10 ml aliquot of 20% sucrose creating a gradient with an interface. Ten ml Aliquots of the spore mixture were then pipetted into the gradient interface and centrifuged at 2500 rpm for three minutes. The layer of VAM spores at the interface was then pipetted onto a 45 mm sieve and rinsed in tap water to remove the sucrose. The spores were then collected onto a Whatman #1 filtre paper under suction using a millipore apparatus. The fungal spores were then picked up individually using a dissecting needle and put either onto a petri dish containing water for observation under a stereo-microscope, or processed into two sided diagnostic slides as described by Schenck and Pèrez (1990) for observation under a compound microscope. Water, PVLA and MPVLA were used as mountants.

3. Multiplication of VAM Fungi Spores

Samples of the collected soils were baited with alfalfa (*Medicago sativa* L cv. Beaver). Approximately 200 g of soil was placed directly beneath but not touching alfalfa seeds in 15 cm pots filled with a double sterilized 3:1 sand:loam mix. These pot cultures were kept in the greenhouse under a 16 hr photoperiod, 500 mE m^{-2} s^{-1} light intensity, day temperatures of 22-24 C and night temperatures of 20 C. Full-strength Hoagland solution, minus phosphorous and adjusted to pH 6.0, was used as fertilizer at 100 ml pot^{-1} week^{-1}, with regular watering. The pot cultures were harvested after six months, plant and root material removed and the soil stored in polythene bags at 4 C until used. VAM fungi spores were isolated from these soils and processed as described above.

4. Identification of VAM Fungi Spores

Spores mounted in water were observed in transmitted light under a stereo-microscope (¥ 50) for color and general morphology of the spores. The diagnostic slides were examined under a light microscope (¥ 100, ¥ 400) and detailed measurements of spore diameter, composite and individual wall thickness, subtending hypha thickness, and width of subtending hypha at point of attachment were taken using an ocular micrometer (Bausch and Lomb). On the basis of these observations, spores were identified through comparisons with VAM fungi descriptions in literature using the guidelines set up by Shenck and Pèrez (1990).

5. Cryopreservation of VAM Fungi Spores

Cultures of *G. intraradices, G. mosseae and G. dimorphicum* were acquired from Dr. J P. Tewari, Plant Science Dept., University of Alberta. *Glomus fasciculatum* was acquired from Dr. N. R. Knowles, Plant Science Dept., University of Alberta. The cultures were multiplied for four months using white clover (*Trifolium repens* L.) as bait. Spores isolated from these cultures were suspended in sterile 20% glycerol (by volume) and centrifuged at 2500 rpm for 3 minutes. The supernatant was discarded and the spores resuspended in sterile 20% glycerol. Surface sterilization of spores was not done. Using sterile pipettes, aliquots of approximately 2 ml were transfered into labelled sterile polypropylene Cryogenic vials. These were then cooled at 4 C for one hour. The vials were then placed on a Handi Freezer tray, (Union Carbide model P/N R036-8C15) The tray was lowered into the neck of Union Carbide 35VHC cryogenic Refrigerator previously filled with liquid nitrogen. The cryotubes remained cooling in the liquid nitrogen for 24 hr at the rate of 6 C $\min^{-1}$. The vapour cooled vials were then loaded on to labelled aluminium canes in a large mouth vessel filled with liquid nitrogen. The canes were then transfered to a Cryogenic Refrigerator filled with liquid nitrogen and stored at -196 C until required.

Seven and thirteen months after storage, (*G. fasciculatum* was only stored for seven months) spores of each species were rapidly thawed in water (35-45 C) until ice crystals were no longer visible. The cryoprotectant was washed off in tap water on a 45 mm sieve. Half of these spores were double autoclaved in a Vernitron Verna-

Clave at 121 C for 30 min. The two sets of spores, autoclaved and unautoclaved were tested for viability using two methods: bioassay and vital stain assay.

6. Vital Stain Assay

The numbers of spores *G. mosseae*, *G. dimorphicum*, *G.intraradices*, and *G.fasciculatum*, were 826, 540, 973, and 960, respectively. The vital stain used was MTT and procedure adopted was that described by An and Hendrix (1988). A stock solution containing 0.5 mg MTT ml^{-1} was prepared with deionized water and stored at 4 C in the dark until needed. Equal volumes of the MTT stock solution and aqueous suspensions of spores were mixed in screw-cap tubes. The caps were closed tightly and incubated in the dark at room temperature for 40 h. The spores were then removed and observed for color changes under a dissecting microscope.

7. Bioassay

This assay was done on spores that had been cryopreserved for 13 months. Thawed spores were collected onto Whatman #1 filter paper and the paper quartered, with each quarter containing fungal spores. Onto each quarter, two surface sterilized alfalfa seeds were placed 0.5 cm away from the VAM spores. The quarters were then folded in a funnel shape, and grown in pot cultures as previously described, for one month.

After one month the roots of each plant were harvested, washed in tapwater, then cleared and stained as described Phillips and Hayman (1970). The roots were first heated in a 90°C oven for one hour in 10% potassium hydroxide. The potassium hydroxide was then poured off and roots were rinsed in tap water until no brown color appeared in the rinse water. Rinsed roots were bleached in alkaline hydrogen peroxide (3 ml ammonium hydroxide, 30 ml 10% hydrogen peroxide, 567 ml tapwater) for ten minutes, rinsed in water and acidified in 1% trypan blue lactic acid staining solution (875 ml lactic acid, 63 ml glycerine, 63 ml tapwater, 0.1 g trypan blue) for five minutes. The staining solution was then poured off and lactic acid destaining solution (same as the staining solution minus trypan blue) was used to remove excess stain.

Spores that were freshly isolated from pot cultures were used as the control.

C. Results

1. Vital Stain Assay

The stain differentiated between viable and non-viable spores of *G. mosseae* and *G. fasciculatum*, but not of *G. intraradices* and *G. dimorphicum* (Fig. IV-0).

In *G. mosseae*, viability of spores was seen to drop from 60% to 16% with seven months cryopreservation, but the vial tested after 13 months cryopreservation showed a 20% spore viability. A small percentage (6%) of autoclaved spores also gave a viable color change (Fig. IV-0).

Viability of spores did not change with storage of *G. fasciculatum*. Again about 7% of autoclaved spores tested viable (Fig. IV-0).

Viability of *G. intraradices* spores were 76% to 85.3% and 86.4% after zero, seven month, and 13 month storage. A high percentage of autoclaved spores (as high as 75.2%) also gave a positive viability reaction (Fig. IV-0).

Viability of *G. dimorphicum* spores was 65.7%, 26.7%, and 56.1%after zero, seven, and 13 months cryopreservation. Autoclaved spores also tested positive for viability: 22%, 64%, and 71% after zero, seven and 13 month storage (Fig. IV-0).

2. Bioassay

The autoclaved spores did not cause any discernible infection in the alfalfa roots. All plants inoculated with non-autoclaved freshly isolated spores had arbuscular infection one month after inoculation, but vesicular infection was not observed. Plants inoculated with non-autoclaved spores that had been cryopreserved for seven months (for *G. fasciculatum*) and 13 months (for the other three species.) all had arbuscular infection (Fig. IV-1). Two of the four plants with *G. fasciculatum* spores also had some vesicular infection (Fig. IV-2). The mycorrhizal entry points were clearly evident (Fig. IV-3) and many of these were observed along infected root lengths (not shown) even for spores preserved for 13 months.

3. Identification of VAM Fungi Spores

Fifteen different spore types were isolated from Kenyan soils. Two of these (type 2 and type 8) were only isolated from the raw field soils collected from Naivasha, but not from greenhouse grown pot culture samples. The rest were all observed in both raw field soil and pot cultures. However, some spores observed in Naivasha soil were not observed in Kiambu soils, while other spores were observed only in Kiambu soils

a. Type one (Fig. IV-4)

In PVLA under transmitted light, spores were a yellow brown color, spherical in shape, and ranged between 21-168 mm. They were a shiny yellow-green in water under incident light. The spores had a simple straight subtending hypha. The composite wall was 9 mm thick and it comprised two wall groups. The outer wall was a brown pitted unit wall approximately 6 mm thick, and the inner wall a yellow brown color and about 2 mm thick. There was no reaction observed with Melzers reagent. The surface of the spore was evenly pitted with the depressions approximately 2 ¥ 3 mm and separated by ridges that were 3 mm across. The pit mouths were circular to ellipsoid. This type of spore was found to be very similar to *Acaulospora scrobiculata* Trappe. This spore type was isolated from soils from both Kiambu and Naivasha.

b. Type two (Fig. IV-5)

Spores were tan in water under incident light. They were globose with a diameter of 213 mm. The composite wall thickness was 12-15 mm thick. Two wall groups were observed, the inner one separating easily from the outer wall. The outer wall was highly ornamented with depressed rings that were approximately 3 mm in diameter. This wall was brown, unit or laminated, with a thickness of 9 mm. Two hyaline walls were observed enclosing spore contents, each approximately 3 mm thick. There was only one hyphal attachment per spore, with a hyphal width of 18 mm at point of stalk. This spore type compared very well with *Acaulospora denticulata* Sieverding & Toro. This spore type was only isolated from Naivasha soils.

c. Type Three (Fig. IV-6)

Spores were subglobose, 132 ¥ 138 mm in diameter, and a dull yellow brown in PVLA. The surface of the spores was covered with spines, except for a funnel-shaped (9 mm ¥ 6 mm) connection to mother the vesicle that was plugged with thickened wall material. The composite wall thickness was 9 mm. A 6 mm outer hyaline wall was separated from the brown-red inner wall by a layer of spines. There was no reaction with Melzers reagent. The spore contents appeared granular. This spore type closely resembled *Entrophospora infrequens* (Hall) Ames & Schneider. This spore type was only observed in Naivasha soils.

d. Type Four (Figs. IV-7, IV-8)

Spores appeared blue in incident light when mounted in water. In PVLA they were hyaline with a lemon yellow tinge, globose, with a diameter range of 95-177 mm. The composite wall thickness was 24 mm and it comprised two wall groups. Group one had two walls, an outer hyaline to brown 3 mm thick wall one and an inner adherent, unit, 0.2-0.4 mm thick wall two. Group two had three hyaline walls. Wall three was ornamented with three-sided arc shaped uniform depressions in side view that were approximately 6 mm long and 3 mm wide, giving the spore surface a scaly appearance. Wall four was 0.25 mm and seemed membranous, and wall five was a rigid, 4 mm thick, unit wall. The spore contents were globular with a globule diameter range of 9-30 mm. In some spores the walls appeared finely laminated. The spores had a straight, thick subtending hypha that was 18 mm wide at the point of attachment, and had 6 mm thick walls. The spores also had two brown scars extending along the outer wall, one 36 mm long and the other 18 mm in a mature spore. This feature was consistent even in young spores. This spore type did not resemble any of the currently documented spore types. Based on the type of hyphal attachment, it was placed in the genus *Glomus*. It was observed in both Naivasha and Kiambu soils.

e. Type Five (Figs. IV-9, IV-10)

Spores appeared opaque under incident light. They were globose with a diameter of 222 mm. The spore contents were between globular and reticulate, white and dense. The spores were hyaline to white with hyaline walls in PVLA but they became a light yellow with greenish yellow walls in MPVLA. The composite wall thickness was 6 mm and comprised three walls in one wall group. In MPVLA, wall one was brown, laminate, and 1.5 mm thick. Wall two was a yellow unit wall, 1.5 mm thick, and wall three was amorphous and approximately 3 mm thick. Two hyphae branched near the spore. The subtending hypha had inconspicuous septation and its wall became red brown in MPVLA. It flared from a straight 6 mm to a funnel shape towards the point of attachment, measuring 12 mm at its widest point. It then constricted gradually to 9 mm at the point of attachment. This spore type resembled *G. lacteum* Rose & Trappe. It was isolated from all pot cultures at very low levels.

f. Type Six (Figs. IV-11 - IV-13)

The spores were a translucent lemon yellow when observed under incident light. There was no reaction with MPVLA. In PVLA, the spore diameter was 78-108 mm. The spores were yellow brown globose, and had granular to globular spore contents. The composite wall thickness was 6 mm thickening to 12 mm at point of hyphal attachment. They had two separable walls, both laminate. Wall one ranged from 4.5-9 mm and was brown, and wall two was of yellow brown color whose thickness ranged from 1.5-3 mm. The subtending hypha was straight to slightly flared, and hyaline to yellow in color. The hyphal walls were 0.5 mm thick and were continuous with the inner spore wall. The pore was 3 mm and occlusion was not observed. This spore type resembled *G. aggregatum* Schenck & Smith emend. Koske and was isolated from soils of Kiambu as well as Naivasha soils.

g. Type Seven (Figs. IV-14, IV-15)

The spores were globose and their diameter ranged from 54 mm. to 144 mm. They were a shiny lemon yellow in incident light and light yellow brown in PVLA. There was no reaction with MPVLA. The composite wall was 12-15 mm thick with five wall layers in one wall group. The outermost wall (wall one) was evanescent, hyaline, and approximately 2.2 mm. The inner walls were all laminate and brown in color, except the innermost one which was a yellowish brown. The spore contents were globular to granular. The subtending hypha was straight, pale yellow to hyaline, 12 mm at point of attachment, and had 3 mm thick walls. This spore type resembled *G. intraradices* and was only observed in Naivasha soils.

h. Type Eight (Fig. IV-16)

There was only one spore observed in this spore type. The spore was formed in a hypha which terminated in a sporiferous saccule 96 mm wide. The spore diameter was 120 mm. It was enveloped in a thin reticulate, hyaline membrane. The reticulations were three sided and arced in side view, 3 mm at their widest point and were separated by ridges 1 mm wide. The composite spore wall was 9-12 mm thick The spore wall below the reticulate membrane was 6 mm with an inner adherent layer that seemed membranous. This spore type was placed in the genus *Entrophospora*.

i. Type Nine (Figs. IV-17, IV-18)

Spores globose, brown to red brown, with a diameter ranging between 24 mm to 220 mm. They only had one wall group comprising one brown unit wall 5-7.5 mm thick. Some spores had an evanescent wall one. The subtending hypha was curved to straight, lemon yellow in color, with a thickness of 15 mm. It flared before the point of attachment to 27 mm, but constricted to 21 mm at the point of entry. The pore was 9 mm and was occluded by a collar-like material. This spore type had strong resemblance with *Glomus deserticola* Trappe, Bloss & Menge. It was isolated only from Naivasha soils.

j. Type Ten (Figs. IV-19, IV-20)

The spores were white with white globular spore contents. They were globose with a diameter of 260 mm. The composite wall thickness was 18 mm and comprised of seven laminate layers that were similar in width. The subtending hypha was hyaline and bulbous based (suspensor-like cell), measuring 34.5 mm at its widest point. It was septate below the suspensor-like cell. The suspensor-like cell had a projection 6 mm wide attached to the spore. This spore type resembled *Gigaspora margarita* Becker & Hall. It was only isolated from Kiambu soils.

k. Type Eleven (Figs. IV-21, IV-22)

The spores were honey gold in color, 309 mm in diameter. The composite wall was 6-9 mm thick and it comprised of one wall group with seven laminated layers. The spores were bulbous based with a suspensor cell 24-27 mm at its widest part, and constricting to 6 mm at the point of attachment to the spore. The spore contents were yellow, granular to globular. This spore type resembled *Gigaspora gigantea* (Nicolson & Gerdmann) Gerdmann& Trappe. It was only isolated from Kiambu soils.

l. Type twelve (Figs. IV-23 - IV-25)

The spores were yellow, spherical to irregular in shape, with a diameter ranging from 60-180 mm. The composite wall was 3 mm with a unit outer wall and an adherent inner wall, which was continuous with the subtending hypha wall. The funnel shaped subtending hypha was 45 mm at the point of attachment. There was a collar, 6 mm thick between the hypha and the spore. The funnel shaped subtending hypha was divided from the spore by a curved septum 33-60 mm down the hyphal attachment. The walls of the sporophore were 3 mm near the point of attachment. This spore type resembled *Glomus mosseae*, and it was only isolated from Kiambu soils.

m. Type Thirteen (Fig IV-26)

The spores formed in brown sporocarps that were ellipsoid, measuring 231 ¥ 180 mm and consisted of a single layer of brown, ellipsoid chlamydospores which averaged 42 ¥ 39 mm in size. The hyphal attachment flared slightly at the point of attachment, averaging 16 mm in width. The composite spore wall was 3 mm, two layered with one wall group, and laminate. The outer layer was dark brown and the inner one was greenish brown. The spore wall thickened towards the subtending hypha to 4.5 mm. This spore type was well described by *Glomus rubiformis* and was isolated from both Naivasha and Kiambu soils.

n. Type Fourteen (Figs. IV-27, IV-29)

Spores were a shiny orange when viewed under incident light. and red brown under transmitted light. They were globose and ellipsoid, 125(-216) ¥ 60-110 mm with a lot of debris on the surface. The spore wall was 6-9 mm, thickening to 9-12 mm towards the point of hyphal attachment. The spores had one wall group consisting of a laminate outer wall which was thicker than the lighter colored unit inner wall. The subtending hypha was simple, straight, and 18 mm thick at point of attachment. It was light yellow with 2.5-3 mm thick greenish walls. The pore contents were granular. The pore remained open. This spore type resembled *Glomus tenebrosum* (Thaxter) Berch. It was isolated from both Naivasha and Kiambu soils.

o. Type Fifteen (Figs. IV-30, IV-31)

The spores were shiny yellow when viewed under incident light. In PVLA mountant the spores were lemon yellow with yellow walls. The outer evanescent spore wall as well as the outer hyphal wall changed color to a reddish brown in MPVLA. The outer spore wall was very weak, breaking away from spore upon the least pressure. The spore diameter was 225 mm with a composite wall thickness of 7.5 mm. The outer wall was a unit wall 4 mm thick and comprised a wall group. The second wall group consisted of a yellow brown unit wall, and an innermost thin, ornamented, unit wall that had projections into the spore. The spore contents were granular. The hyphal attachment flared from 15 mm to 30 mm at the point of attachment. The spore contents formed a curved collar 15 mm down the subtending hypha. The pore was occluded by a collar that was continuous with the inner spore wall. The subtending hypha was thin walled, circa 0.6 mm, and was aseptate. This spore type resembled *Glomus caledonium* (Nicolson & Gerdemann) Trappe & Gerdmann and was only isolated from Kiambu soils.

D. Discussion

1. Viability Testing

The MTT vital stain failed to give typical reactions with two of the VAM species tested. This would lead one to conclude that the *G. dimorphicum* and the *G. intraradices* spores were resistant to the moist heat they were subjected to during autoclaving. However, autoclaved spores of these species did not infect alfalfa plants, indicating that they were dead. Spores of VAM have been found to be susceptible to moist heat. Tommerup and Kidby (1980) found that neither *Glomus caledonius* nor *Acaulospora laevis* retained their viability after exposure to 60 C moist heat for five minutes. The phenomenon of autoclaved spores giving a positive viability reaction with MTT has also been observed for *Phytophthora* species oospores and could not be explained (Sutherland and Cohen, 1983). There is need therefore for rigorous studies of the correlation of spore stainability with germinability before a protocol for using vital stains to test for viability can be set up.

The increase of percent viable spores with cryopreservation portrayed by the VAM species tested was an artifact. Different vials of spores may have contained different amounts of viable spores before they were preserved. Thus this experiment can only be interpreted qualitatively and not quantitatively.

An and Hendrix (1988) indicated the MTT procedure gives a reliable estimate of viability for Endogonaceous spores isolated from field soils. The results of this study showed clearly that the MTT procedure should not be used indiscriminately to test all species of VAM fungi for spore viability. The efficacy of vital stains in viability tests differs among the stains for different plant species (Bulat, 1972), a phenomenon that may be carried over to fungal spores. Given the results of the present study, although the vital stain procedure does have great potential for quantitative determination of VAM spore viability, specific techniques may be needed for the different species. One should also keep in mind that VAM fungal spores have a high propensity for hyperparasitism, (Daniels and Menge, 1980) and that a parasitized dead VAM spore may therefore test viable. There is a need for a reliable, repeatable method to quantitatively determine viability of VAM fungal spores of different species.

Although no quantification of root infection was done, the numerous points of infection observed on roots of plants inoculated with revived cryopreserved VAM fungal spores indicated that a large percentage of the spores retained their viability, even after 13 months of cryopreservation. In the absence of quantitative information, it was difficult to determine by how much spore viability was affected by cryopreservation. This study showed that cryopreservation in liquid nitrogen may offer an alternative space efficient, low technical know how method of preserving VAM fungal spores.

2. Identification of Fungi Spores

Acaulospora denticulata and *Enthrophospora* sp. were not isolated from pot cultures. This may have been because they did not multiply in culture, or if they did, they were too few and were therefore not present in the samples observed. Failure to multiply may have been due to dormancy (Tommerup, 1983). It may also have been due to outcompetition by other VAM fungi since these were mixed cultures (Hepper et al. 1988). There are disagreements about the limiting factors that the VAM fungi compete for, but space and nutrients seem to be the most likely factors (Wilson and Tommerup, 1992). Infectivity of spores is known to differ both amongst (Thomson et al., 1990) and within species. Wilson (1984a) showed the differences in the infectivities of *Glomus fasciculatum*, *G. tenue*, and *Gigaspora decipiens* depended on inoculum density, implying intraspecific competition. Another explanation for failure of the spores to multiply could be that they needed association with certain micro-organisms which were absent in sterilized soils. Tylka et al. (1991) showed that three *Streptomyces* sp. stimulated germination of *Gigaspora margarita* and *Glomus mosseae* spores.

The rest of the spore types all multiplied in pot cultures. The red reaction of the innermost spore wall of *Acaulospora scrobiculata*, combined with the light colored pitted spore wall distinguish it from other *Acaulospora* species. The spores isolated in this study that resembled *Acaulospora scrobiculata* did not show the Melzers reaction, but all other spore characteristics matched those of *Acaulospora scrobiculata*

Very few spores of type two were observed, indicating the low aggressive nature of that VAM fungus. The spore size and wall characteristics resembled those of *Acaulospora denticulata* strongly. The lack of a cicatrix was not surprising because on species of *Acaulospora*, ciatrices are difficult to discern (Schenck and Pérez, 1990). There was one spore that had a short hypha.

The identification of the third spore type was based on spore wall characteritics since subtending hypha was absent. A plugged up funnel-shaped connection to the mother cell was however present. The projections arising from the inner wall differentiated these spores from spores of other species. There was no known spore type that resembled spore type four. Both young and older spores were observed and it did not seem likely that these were juvenile or parasitized spores. It is highly likely that this could be a new spore type that has yet to be described.

Although spore type five was best described by *G. lacteum*, the spore wall characteristics did show some discrepancies. *G. lacteum* is reported to have only one layer of wall, is white in color, and frequently exhibits multiple hyphal attachments. Spore five had three layers of wall with one being amorphous. Spore five was differentiated from *Glomus albidum* Walker & Rhodes by its larger sized spores, its smooth wall surface that did not slough off, and its persistent white color. This spore type seemed to have characteristics of both *Glomus albidum* and *G. lacteum*, but resembled the latter the most. This spore type was isolated from all pot cultures, but it was not abundant.

Spore type six displayed all the characteristics of *Glomus aggregatum*, and did not react with Melzers reagent, thus differentiating it from *Glomus fasciculatum*. This was the most abundant spore type in the Kiambu soils and it multiplied very vigorously in pot cultures making identification much easier. For Naivasha soils, spore type seven was the most abundant, and it multiplied vigorously in pot cultures. This spore type had a characteristic ephemeral outer hyaline wall and laminated walls that gave the hyphal attachment a characteristic tubaeform flare. The spore type nine resembled spore type fourteen except that it had red brown spores, young and old, and it had a one layered composite wall. The deep reddish brown walls differentiates *G. deserticolo*r from *G. fasciculatum*.

Spore type ten had typical *Gigaspora margarita* characteristics, except that under pressure the spore wall separated to give two wall groups which is not characteristic of *G. margarita*. However, this could have been an artifact brought about by too much pressure applied to the spore. Not many spores were available for comparison purposes. Spore type eleven had spores that were actually smaller than typical *G. gigantea*, and more like the size of those described Mosse and Bowen (1968) from New Zealand and Australia, which they equated with *G. gigantea*. Gerdmann and Trappe (1974) did not think that they belonged to *G. gigantea*, It is possible that this spore type comprises a smaller spore strain of the fungus.

Spore type twelve was prolific in culture. They were identified as a strain of *Glomus mosseae* mainly on the basis of their flared subtending hypha. The identification of spore type fourteen was tentative, as its spores are much smaller than those described as *G. tenebrosum*. The identification of type fifteen was also tentative.

The described spores show that there is a variety of VAM fungi associated with the carnation growing areas of Kenya. This was not surprising since the carnation culture is mainly practiced under a mixed farming system involving crops like maize, beans, coffee, bananas, forages, pear trees, potatoes (sweet and irish), and other flowers like marigold and statice. The carnations are usually grown in rotation with these crops. This means there is a good indigenous pool that can be used to select VAM fungi that may most efficiently enhance crop production. It would be prudent to consider the possible interactions these fungi would have with any other VAM fungi species introduced to these soils.

E. Figures, Tables and Legends

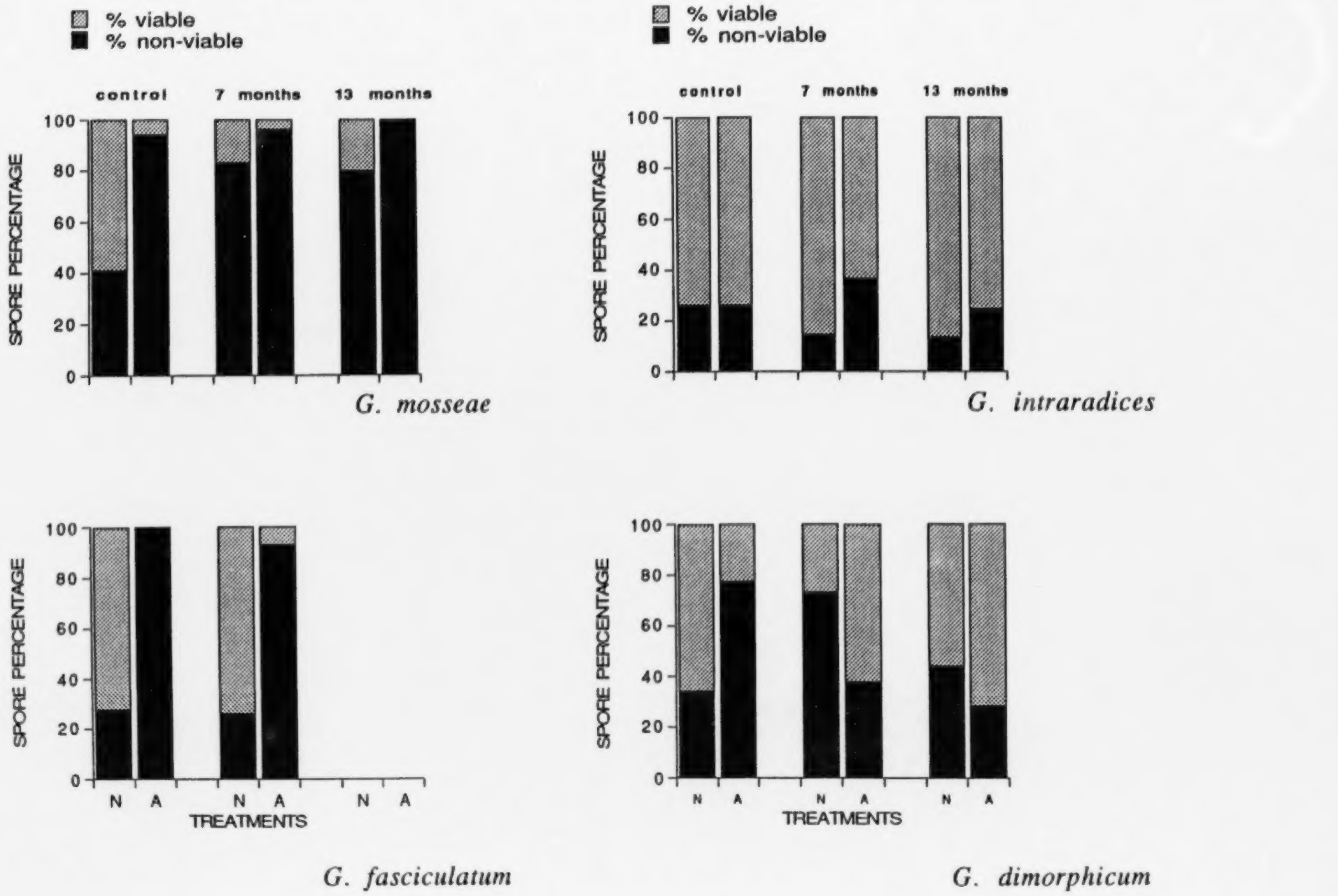


Fig IV-0. The effect of cryopreservation on the viability of VAM fungi spores, as tested using MTT vital stain, comparing autoclaved (A) and non-autoclaved (N) spores.

Fig. IV-1. Alfalfa root cells with highly branched arbuscules of VAM spp. that had been cryopreserved for 13 months in liquid nitrogen (× 500) (hollow arrows)

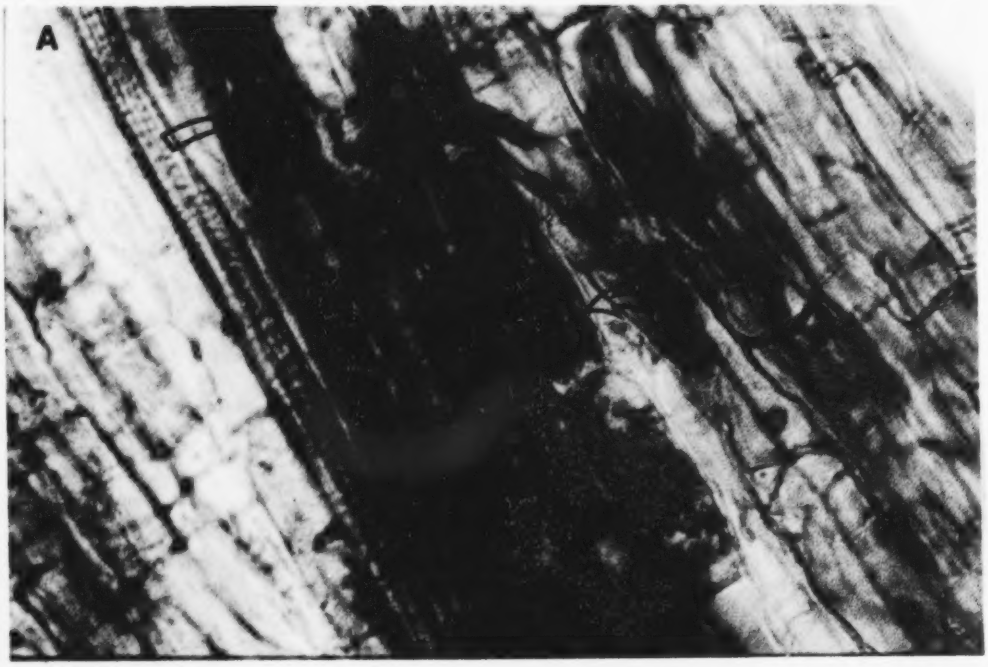

A

Fig. IV-2. Alfalfa root cells infected by *Glomus fasciculatum* (× 500)that had been cryopreserved for seven months in liquid nitrogen

A Vesicles (small arrowhead). Note the point of infection (open arrow)

B Arbuscules (arrowhead) and vesicles (small arrowhead)

C Secondary spread of VAM infection within the root. Infection points along the intraradical hypha (arrows)

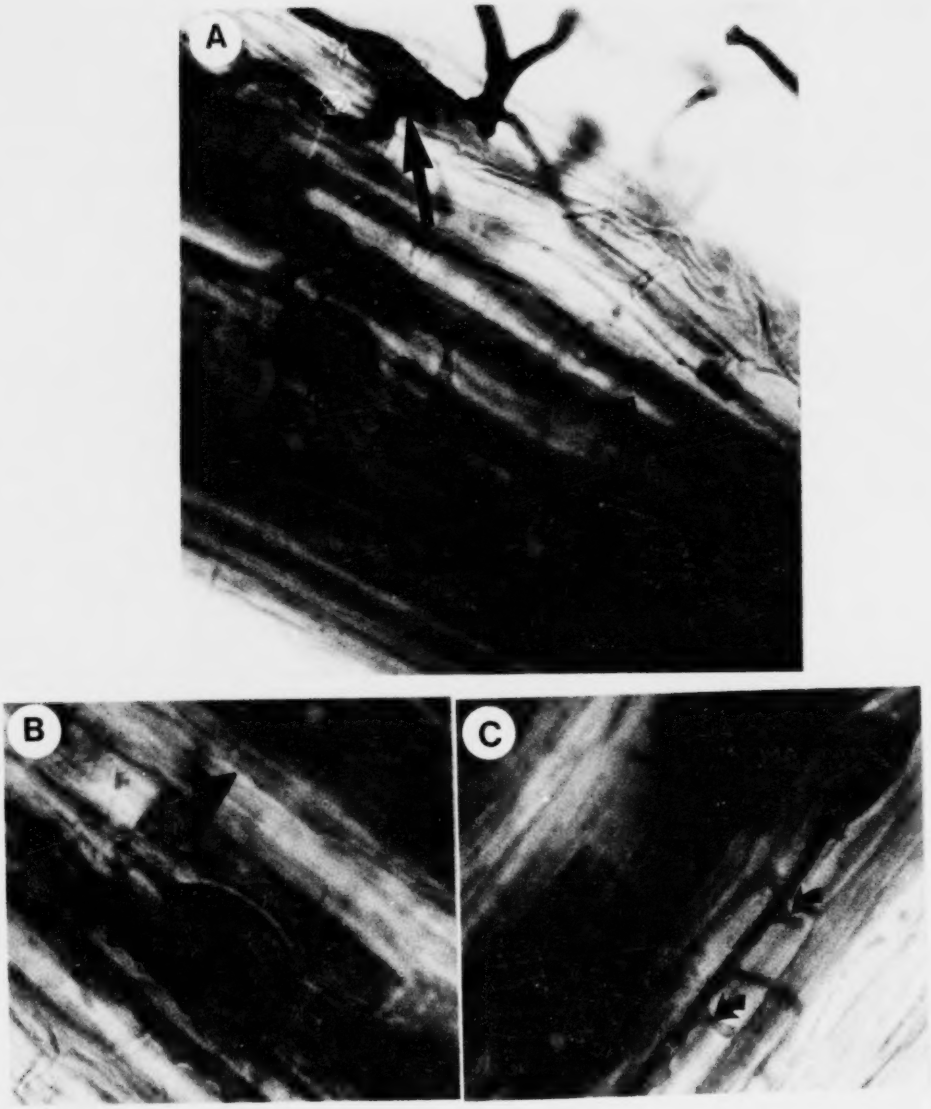

A
B
C

Fig. IV-3. Infection of alfalfa roots by VAM fungi spores that had been cryopreserved in liquid nitrogen for 13 months (× 500)

A Multiple infection points (arrows) from VAM hyphae at the root surface. Note appressoria (arrows) and the dense arbuscules within the root

B Details of the infection point showing the infection peg (arrow) and appressorium (arrowhead)

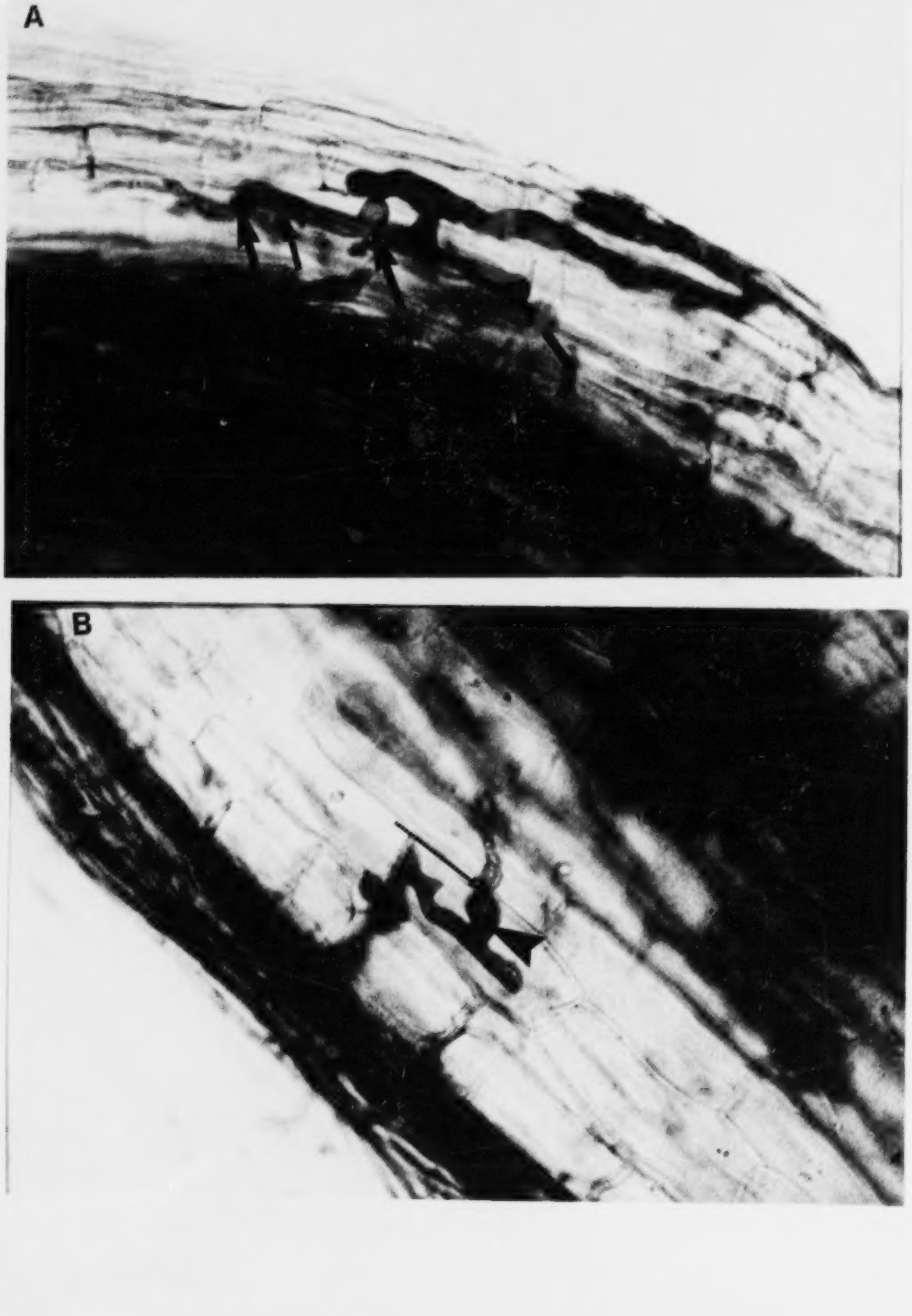

A
B

Fig. IV-4. Light micrograph of *Acaulospora scrobiculata* spores isolated from soils from carnation growing areas of Kenya
Note the evenly pitted dark outer spore wall
Notice hyphal stalk exuding lipid droplets from cut surface

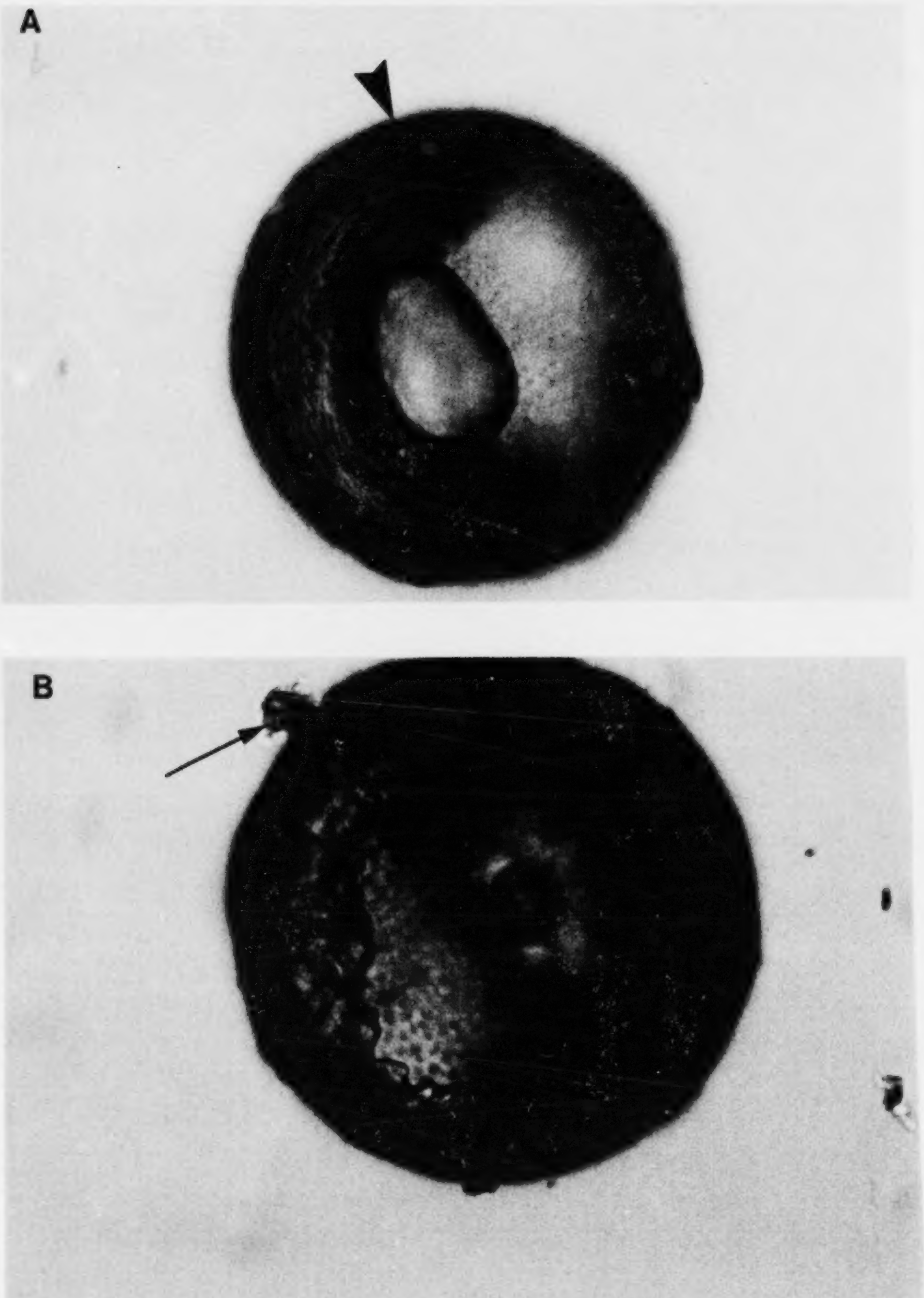

A
B

Fig. IV-5. Light micrograph of *Acaulospora denticulata* spores isolated from soils from carnation growing areas of Kenya

A Note the hyphal attachment scar (arrow)

B Note wall group I (arrowhead) and two hyaline walls of group II (arrow)

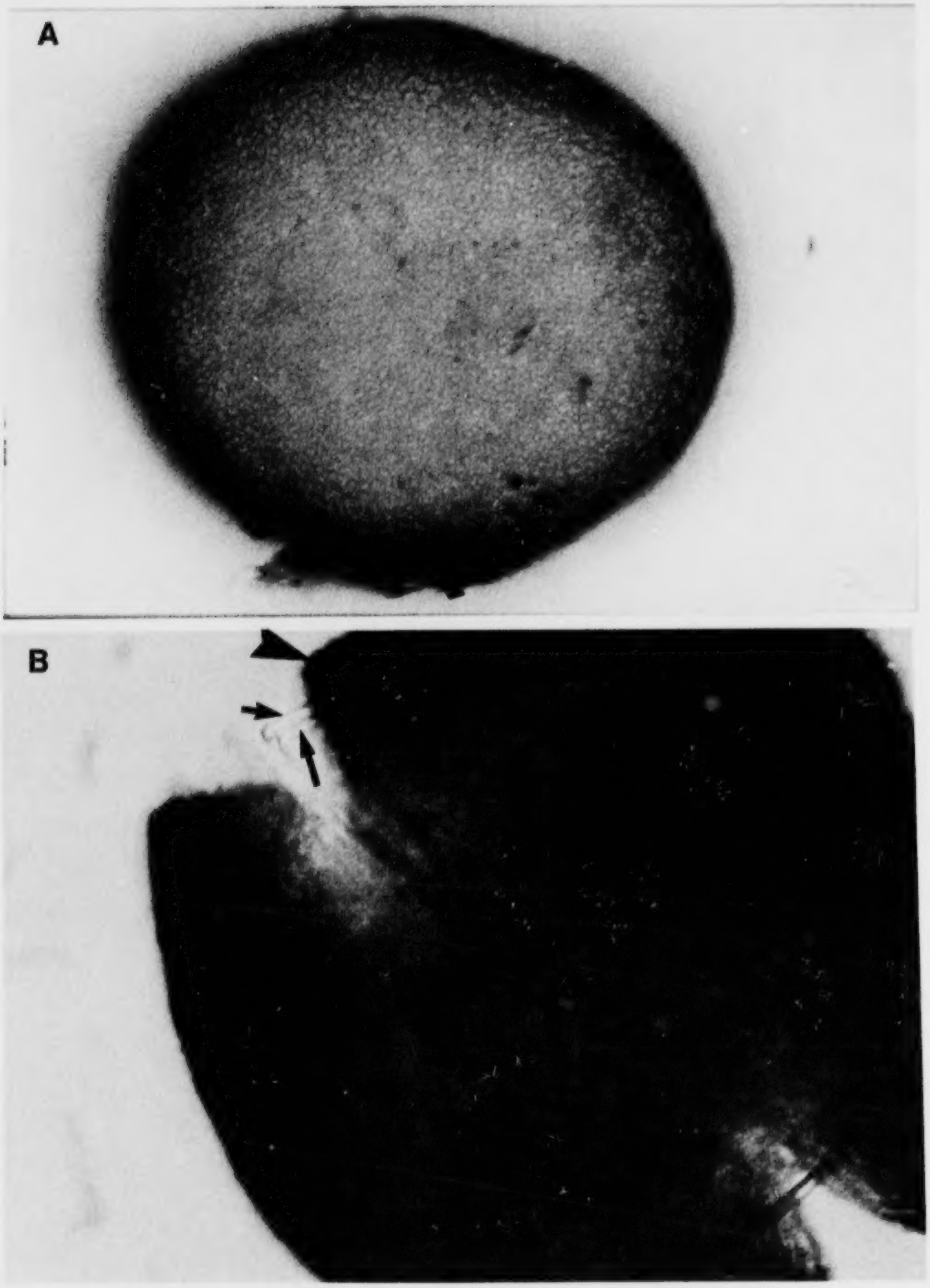

A
B

Fig IV-6. Light micrograph of *Entrophospora infrequens* spore (x560) isolated from soils from carnation growing areas of Kenya

A Note funnel-shaped plugged connection to mother vesicle (slender arrow)

B spines separating the outer hyaline wall from the inner brown wall (slender arrow), and the spore within the spore (curved arrow)

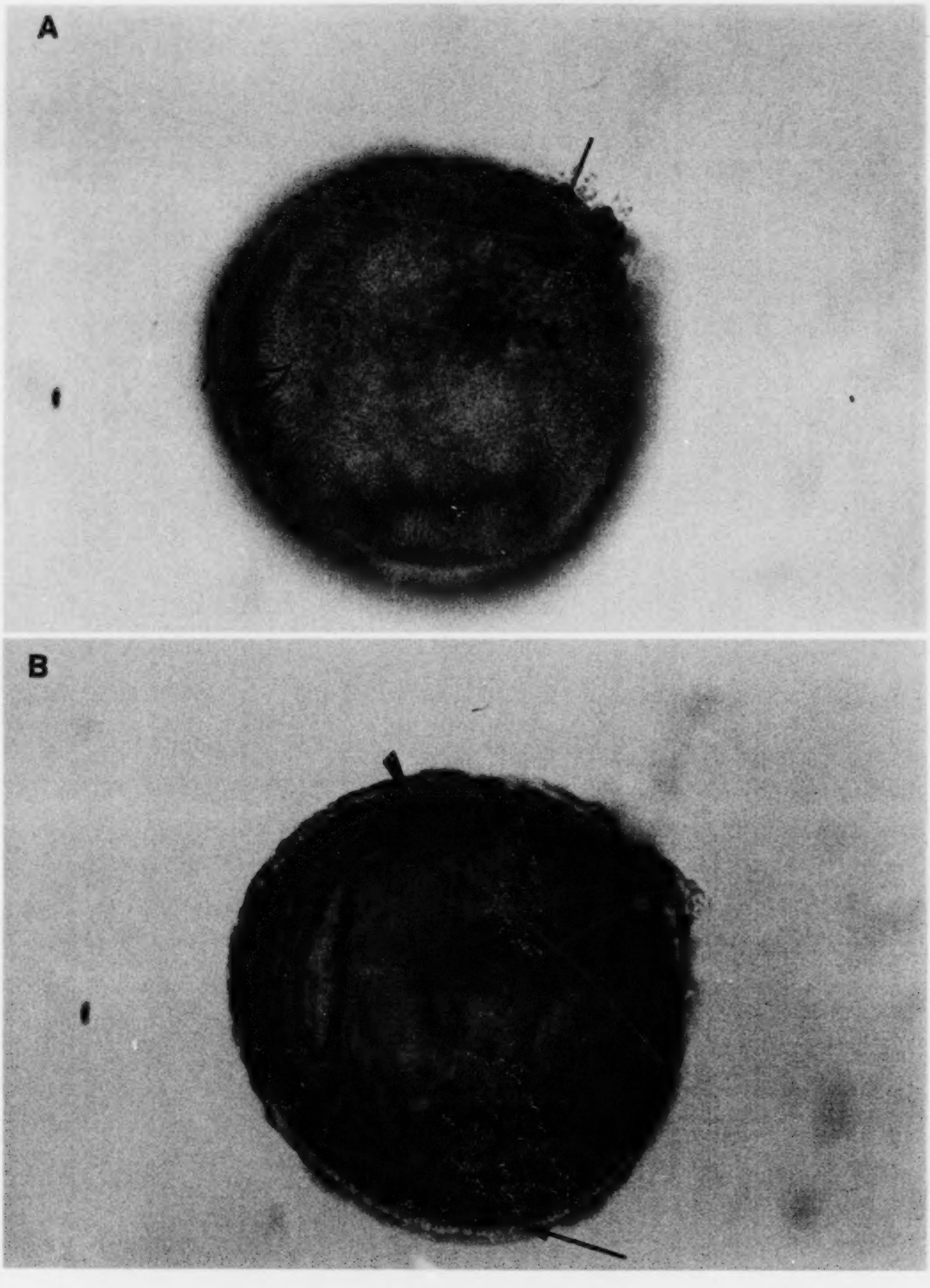

A
B

Fig. IV-7. Light micrograph of a *Glomus* sp. spore isolated from soils from carnation growing areas of Kenya

A (x508) Hyline multilayered spore. Note the two brown scars along the outermost wall (arrowheads)

B (x850) Note the ornamental wall III (arrow) and the globular spore contents (arrowhead)

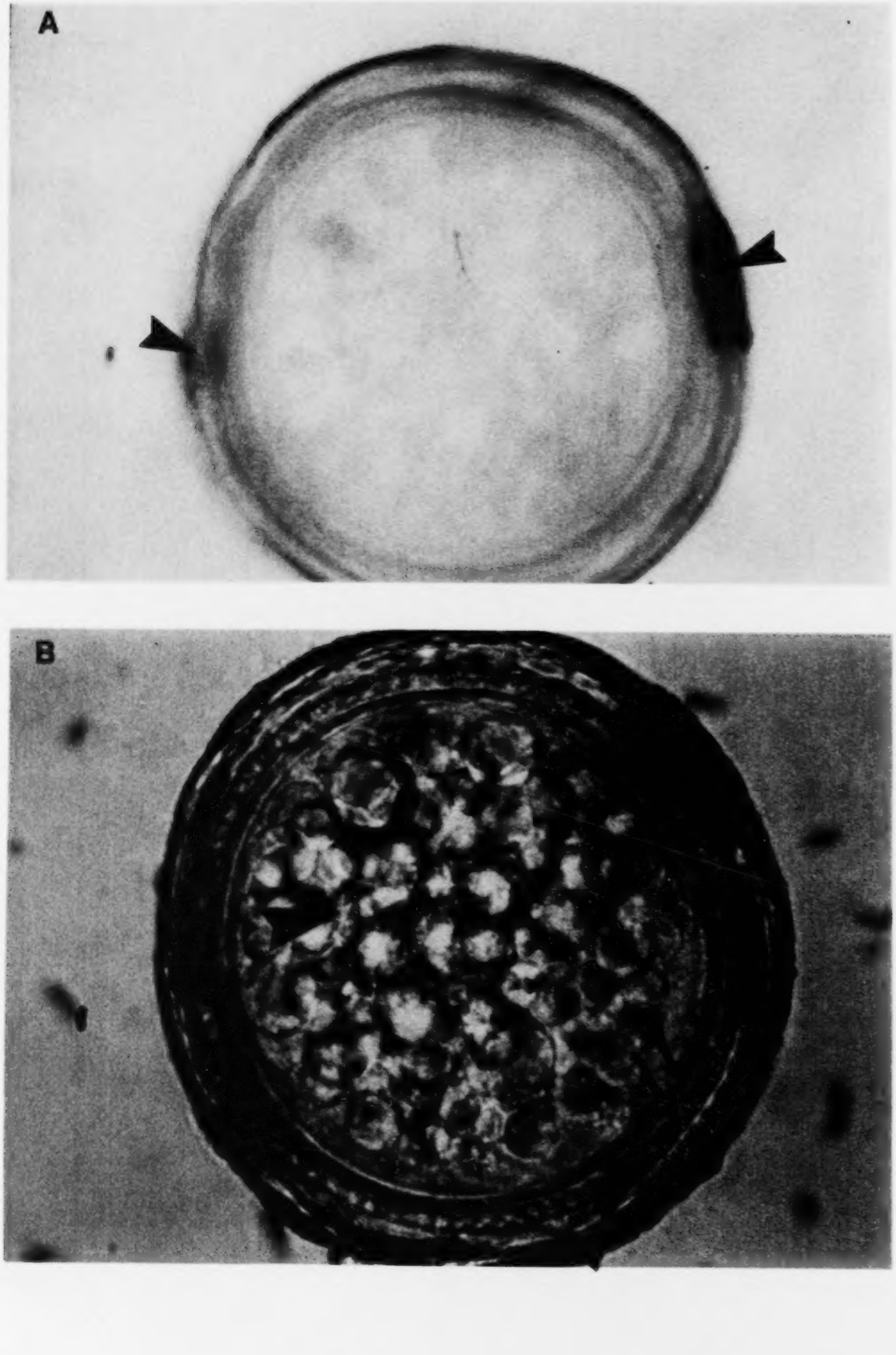

A
B

Fig. IV-8. Light micrograph of a *Glomus* sp. spore isolated from soils sampled from carnation growing areas of Kenya

A Details of hyphal attachment. Notice the thick hyphal walls continuous with outer spore wall (arrows)

B. (x897) Details of ornamentation on wall III. Note the three-sided, arc-shaped depressions (arrow)

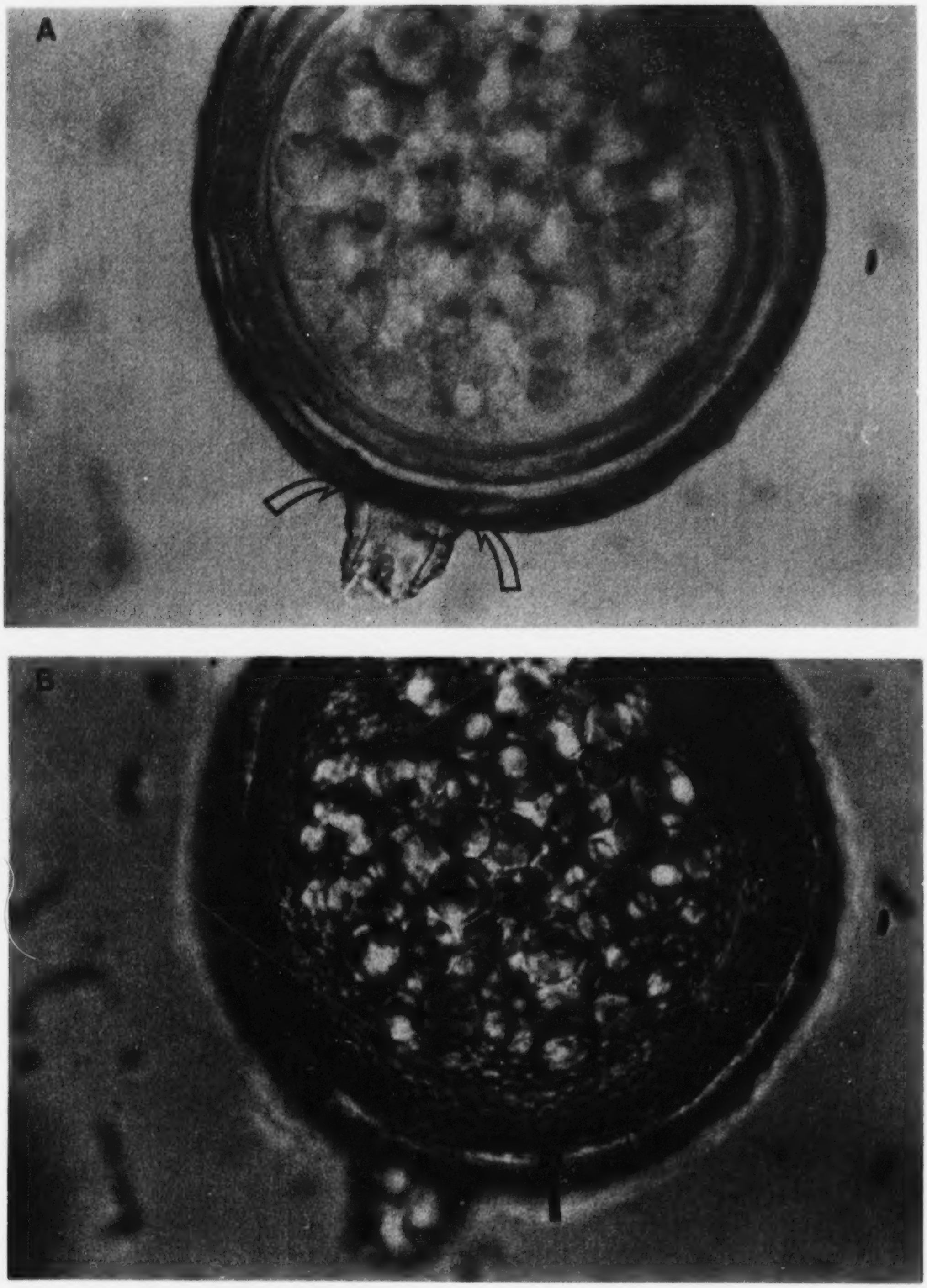

A
B

Fig. IV-9. Light micrograph of *Glomus lacteium* isolated from soils sampled from carnation growing areas of Kenya

A (x135) Note the three hyphae branching from one subtending hypha (arrowheads) and the red-brown colouration on the subtending hypha (arrow)

B (x400) A younger spore in PVLA. Note the milky-white spore contents (star) and hyaline outer wall (arrow)

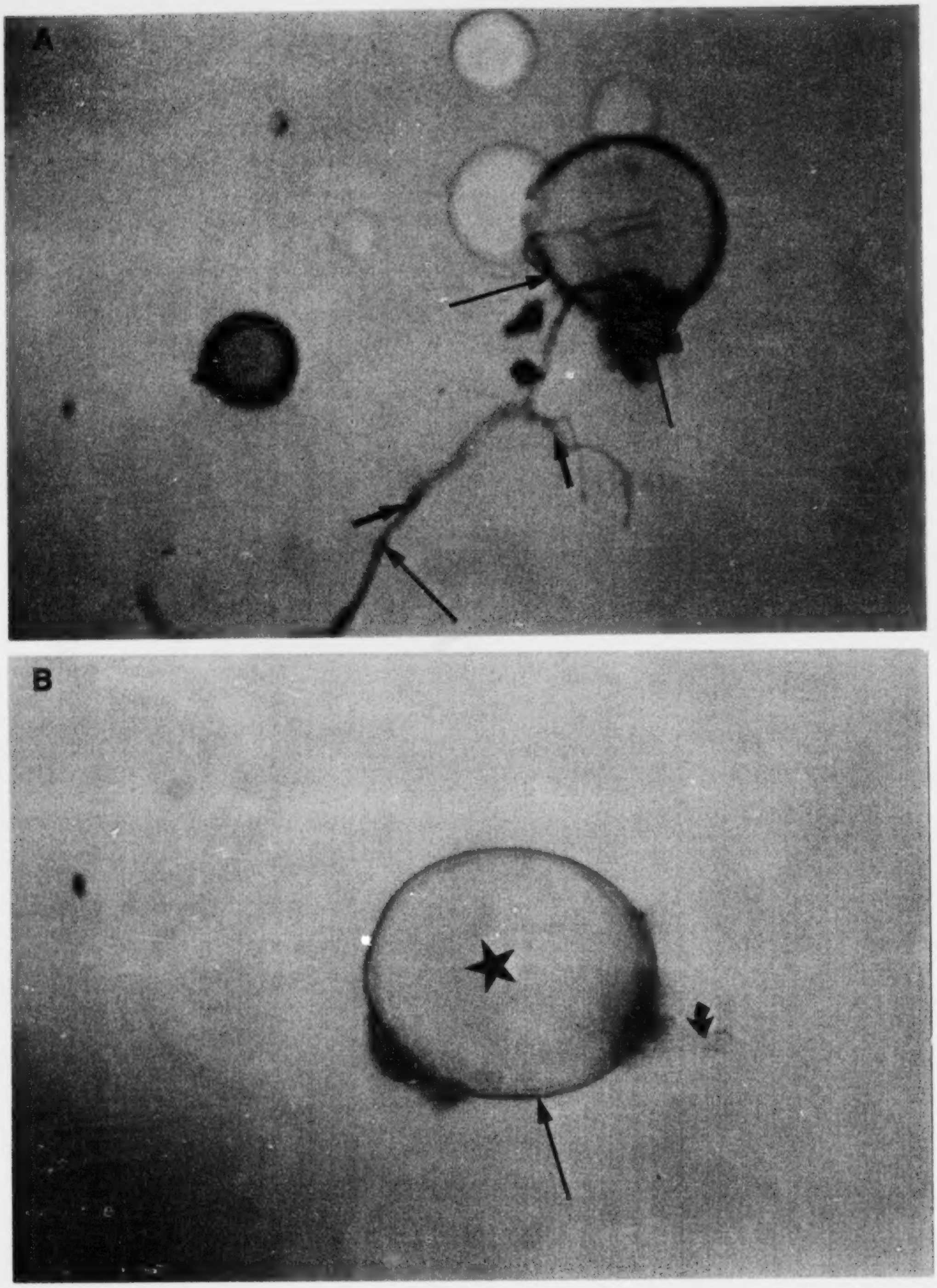

A
B

Fig. IV-10. Light micrograph of *Glomus lacteium* isolated from soils sampled from carnation growing areas of Kenya

A (x405) Notice two hyphae branching from one attachment (curved arrows). Note the red-brown stain (slender arrows) and the flared hyphal attachment (large arrowheads)

B Higher magnification of spore in A. Note the amorphous wall III (arrow) and the oil droplet (arrowhead)

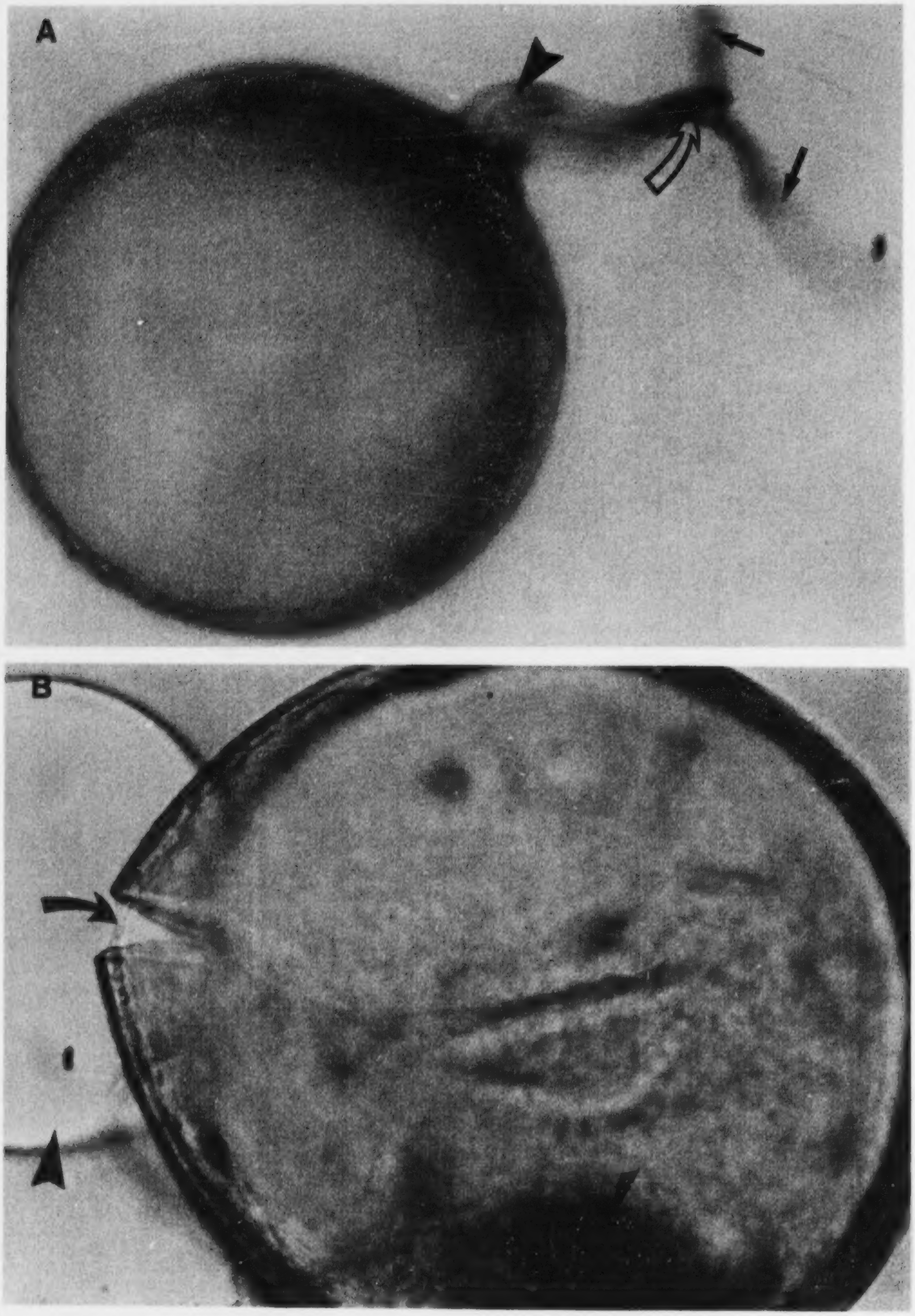

A
B

Fig. IV-11. Light micrograph of *Glomus aggregatum* (x100) isolated from soils sampled from carnation growing areas of Kenya

A Sporocarp

B Note the outer dark laminate wall (arrow) and the inner, lighter wall (arrow)

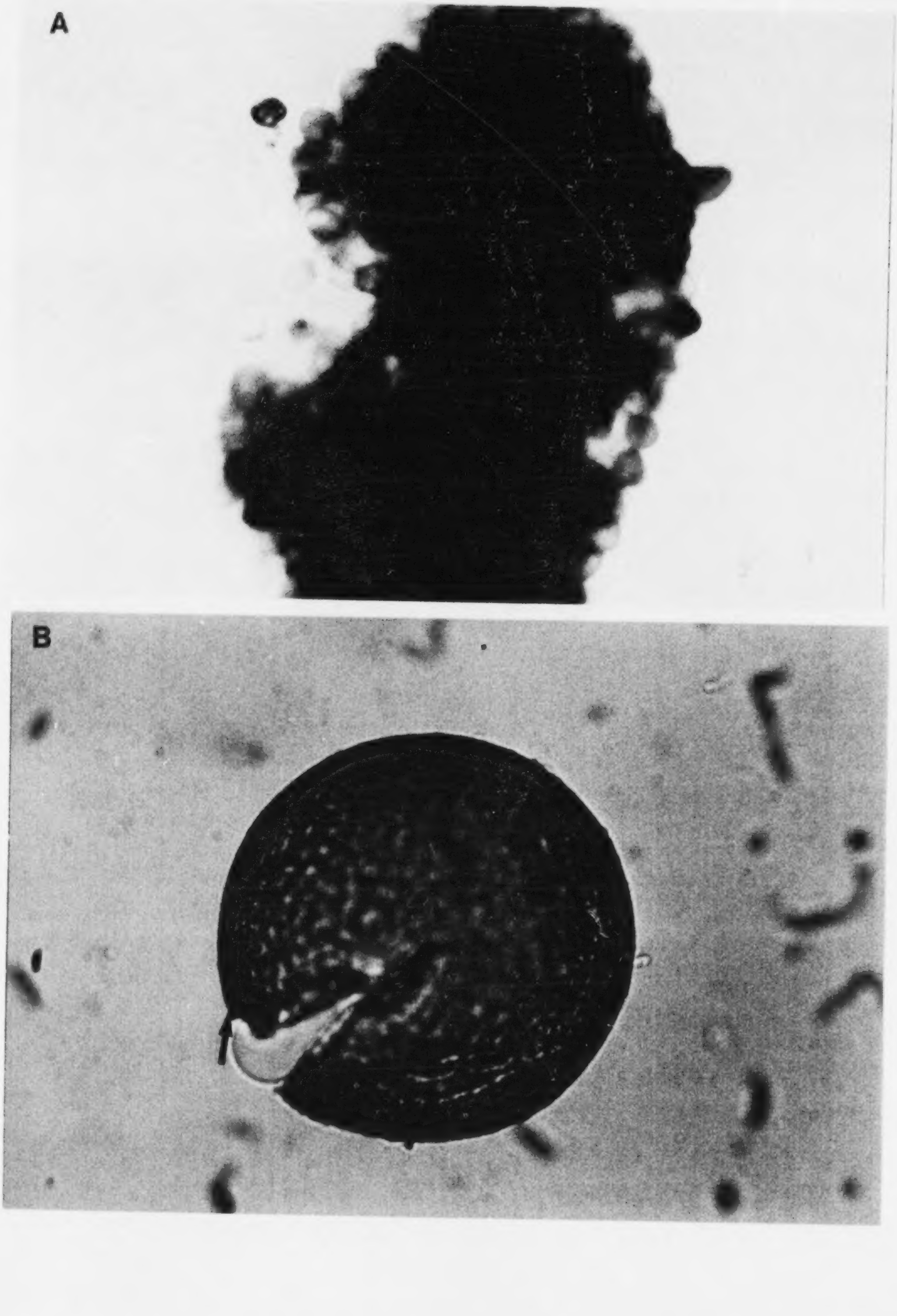

A
B

Fig. IV-12. Light micrograph of *Glomus aggregatum* isolated from soils sampled from carnation growing areas of Kenya

A (x700)Note the branched subtending hypha (arrowhead)

B (x750) Note the open pore (arrow)

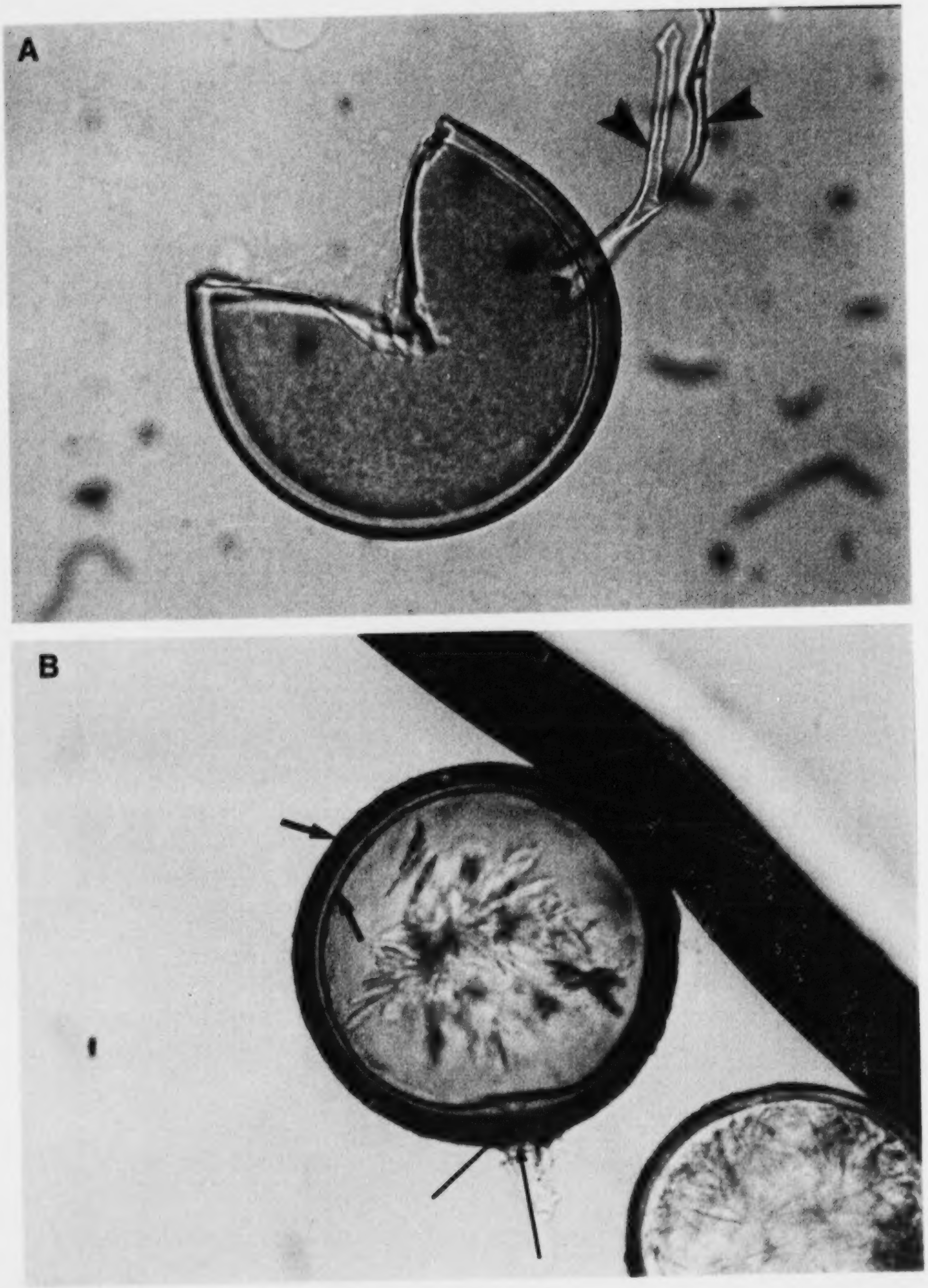

A
B

Fig IV-13. Light micrograph of *Glomus aggregatum* isolated from soils sampled from carnation growing areas of Kenya

A (x633) Note the hyaline, thin outer wall with a greenish tint (arrowhead)

B (x650) Note the evanescent wall sloughing off (arrowhead)

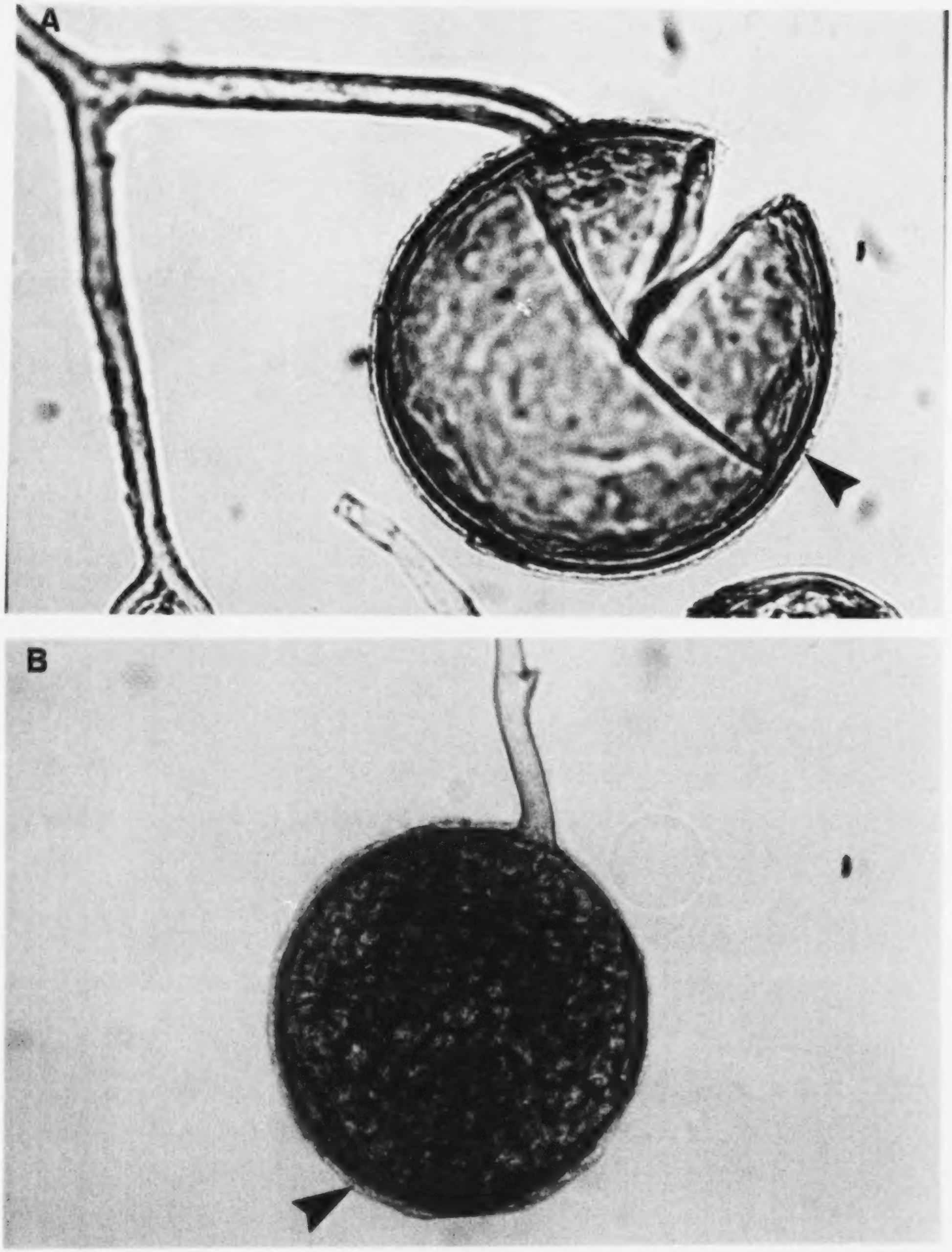

A
B

Fig IV-14. Light micrographs of *Glomus intraradices* spores isolated from soils sampled from carnation growing areas of Kenya

A (x1000) Note the hyaline outer wall of the spore (arrowhead) as well as the proliferation of a spore within another spore (arrow)

B (x555) Notice the slight flair of the hyphal attachment (arrow) and the straight subtending hypha (curved arrow)

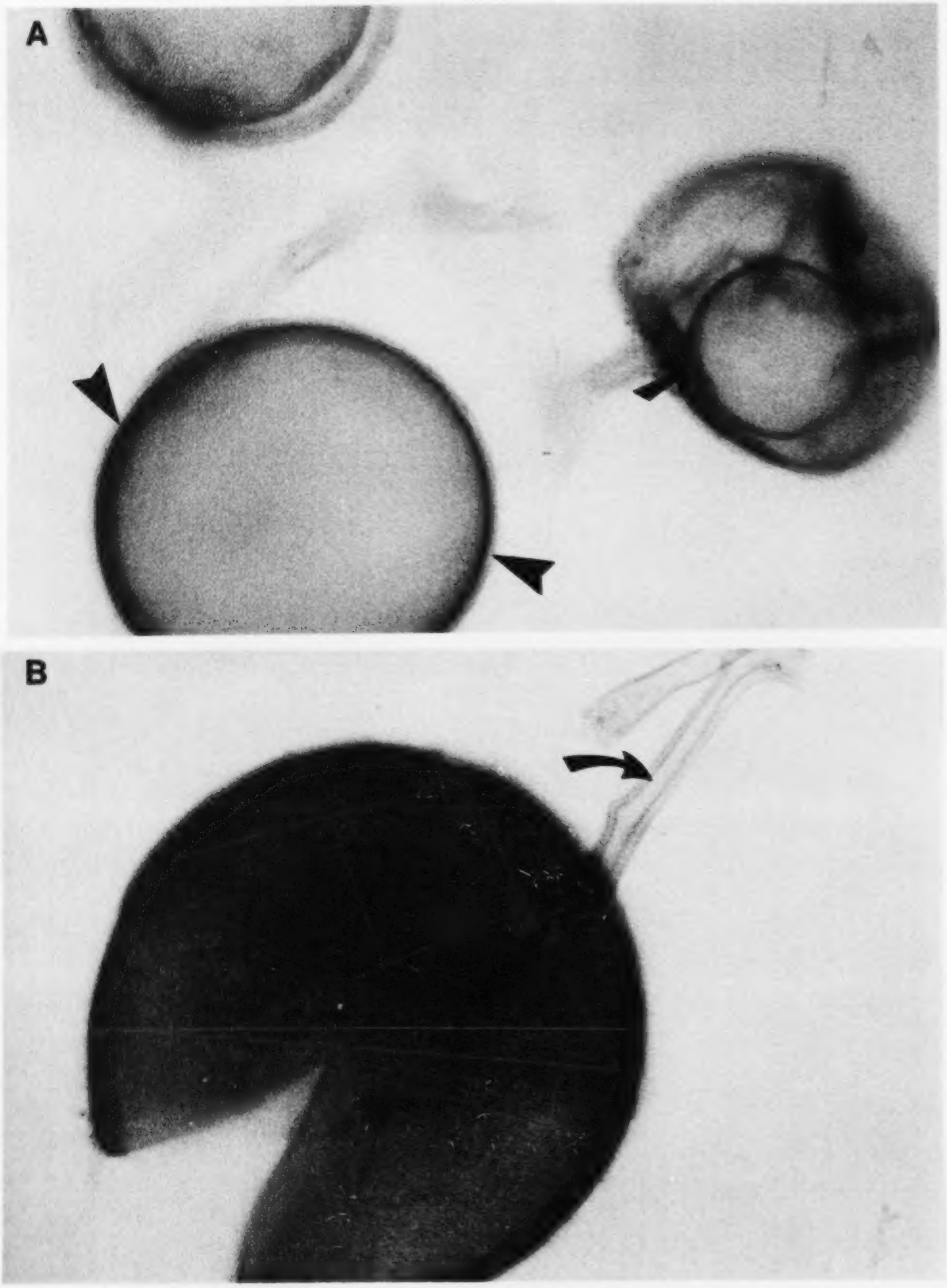

A
B

Fig IV-15. Light micrographs of *Glomus intraradices* spores isolated from soils sampled from carnation growing areas of Kenya

A (x560) Note the laminate spore wall (arrowhead)

B (x600) Note the ephemeral outer wall sloughing off

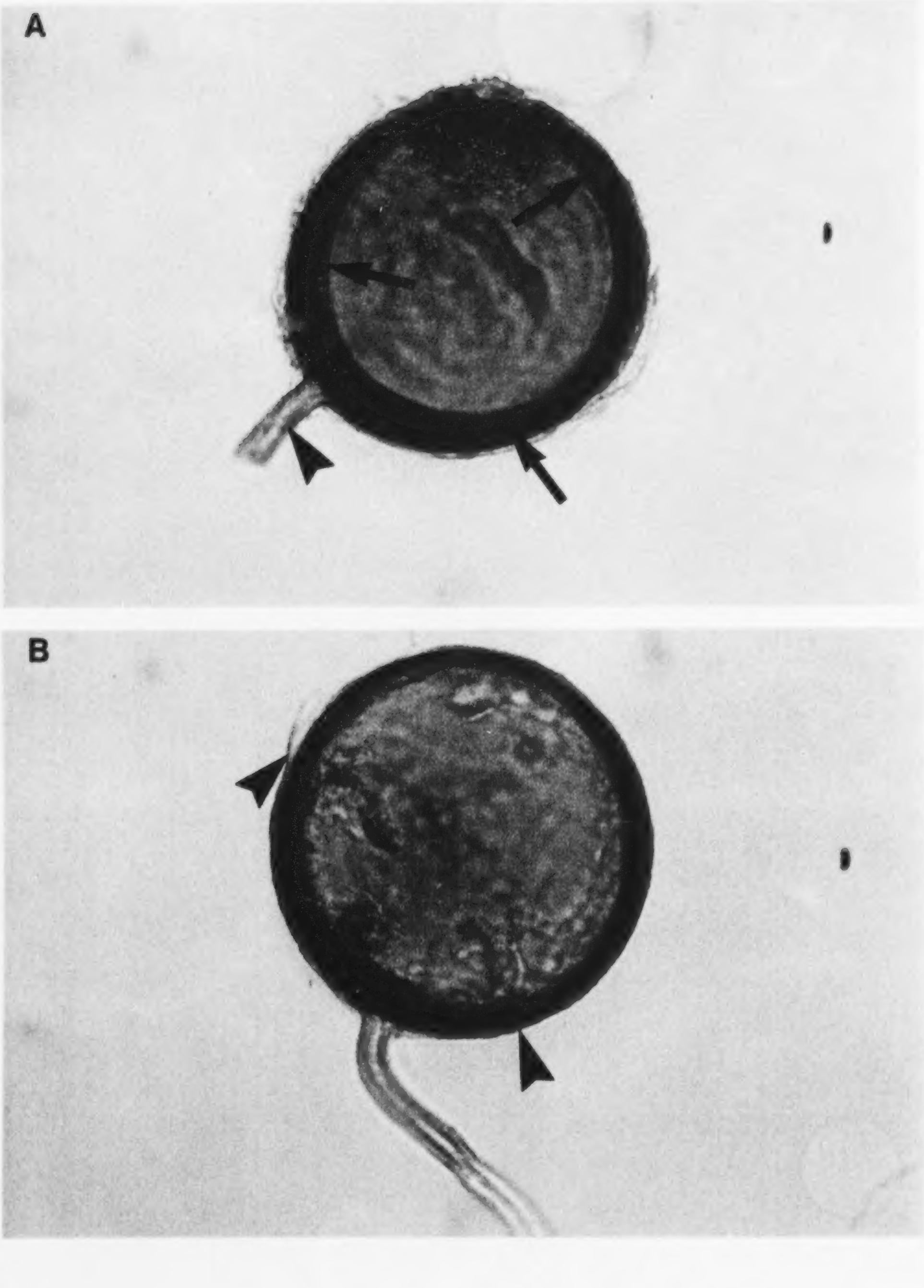

A
B

Fig. IV-16. Light micrograph of *Entrophospora* sp. (x583) isolated from soils sampled from carnation growing areas of Kenya

A Note the dark outer spore wall (arrowhead) and the collapsed sporiferous saccule (arrow)

B Note the reticulate hyaline membrane (arrows)

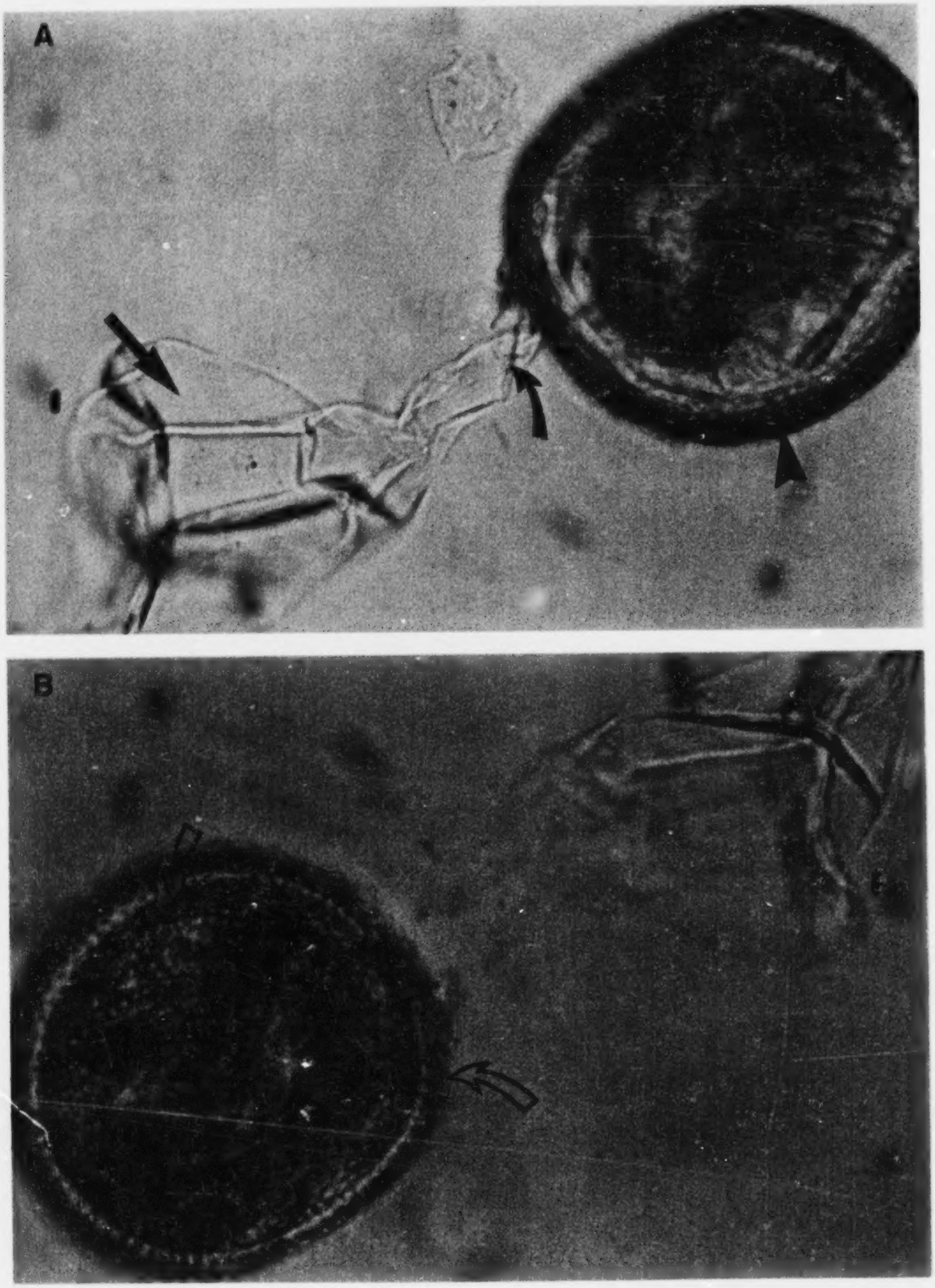

A
B

Fig. IV-17. Light micrographs of *Glomus deserticola* spores isolated from soils sampled from carnation growing areas of Kenya

A Young spores (x1400)

B Mature spore (x450) Notice the single wall layer (arrowheads) and the light coloured tubular sporophore

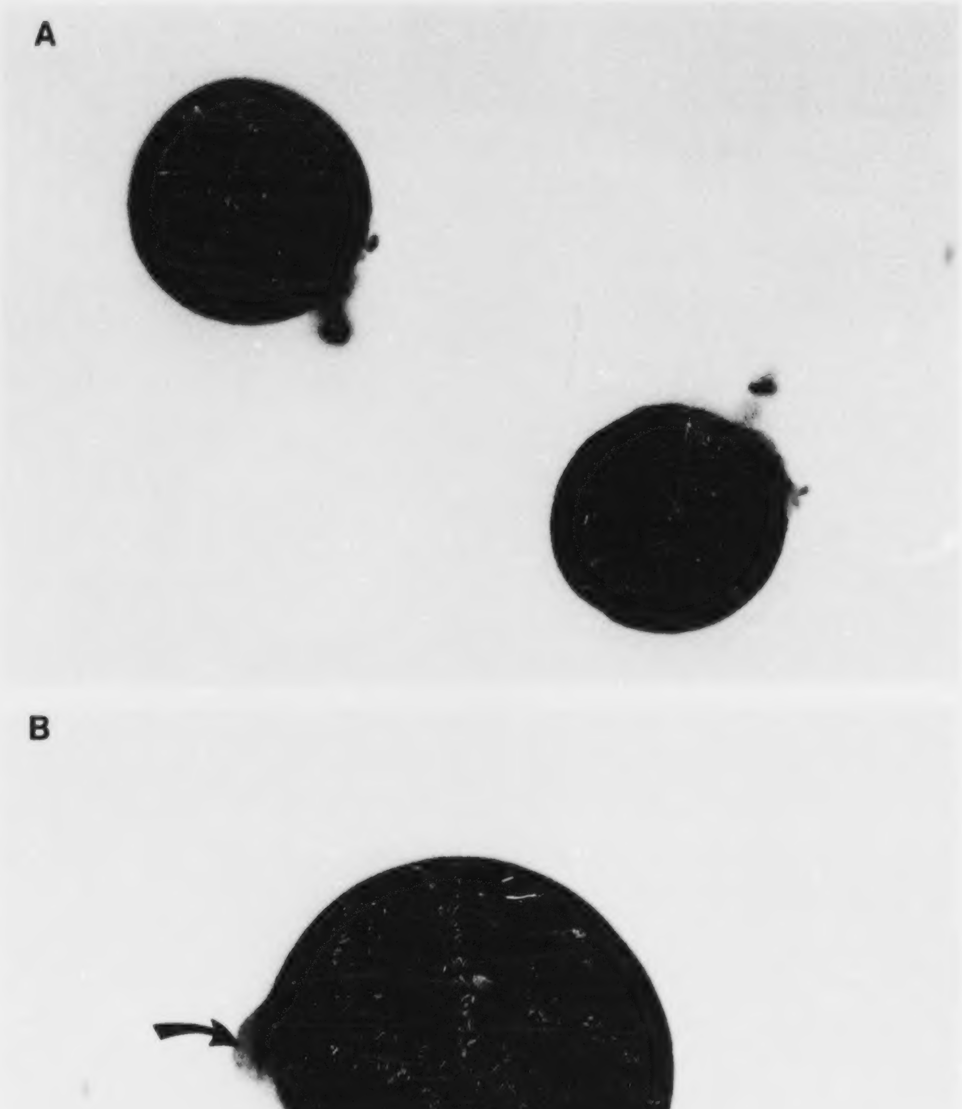
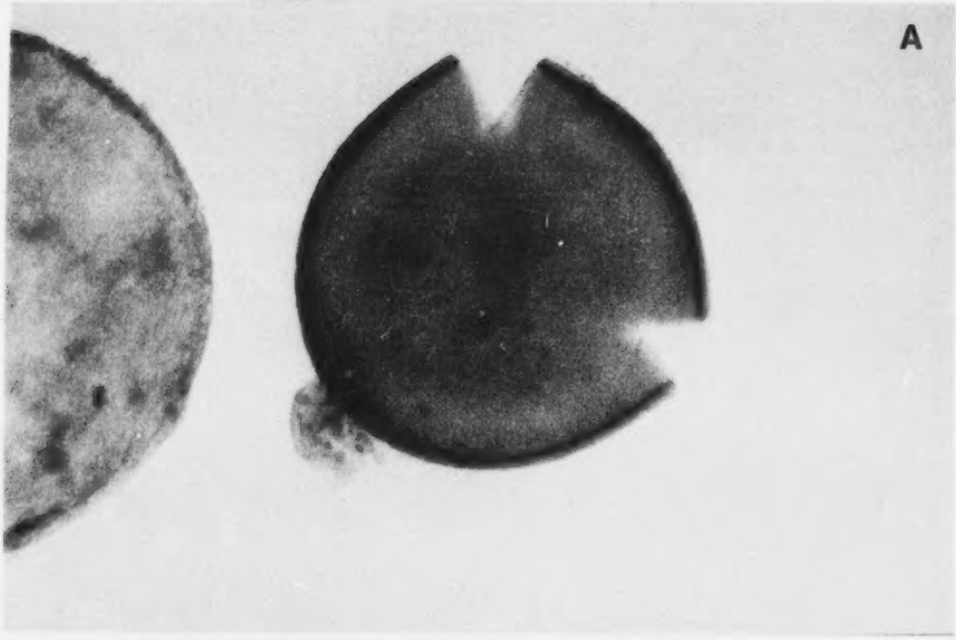

A
B

Fig. IV-18. Light micrographs of *Glomus deserticola* spores isolated from soils sampled from carnation growing areas of Kenya

A (x450) Lighter-coloured spores

B Parasitization by some VAM fungi spores (arrow)


A

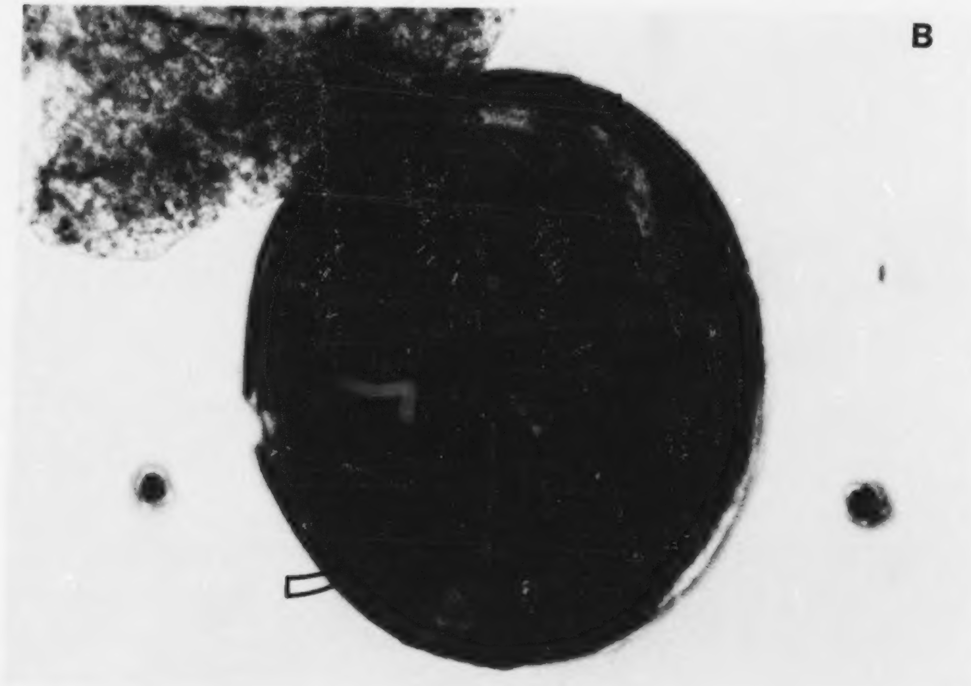

B

Fig. IV-19. Light micrograph of *Gigaspora margarita* spores isolated from soils sampled from carnation growing areas of Kenya

A (x115) Notice the green tinge of spore wall and contents (arrows)

B (x538) Note the globular spore contents (star) and the laminate walls (arrows)

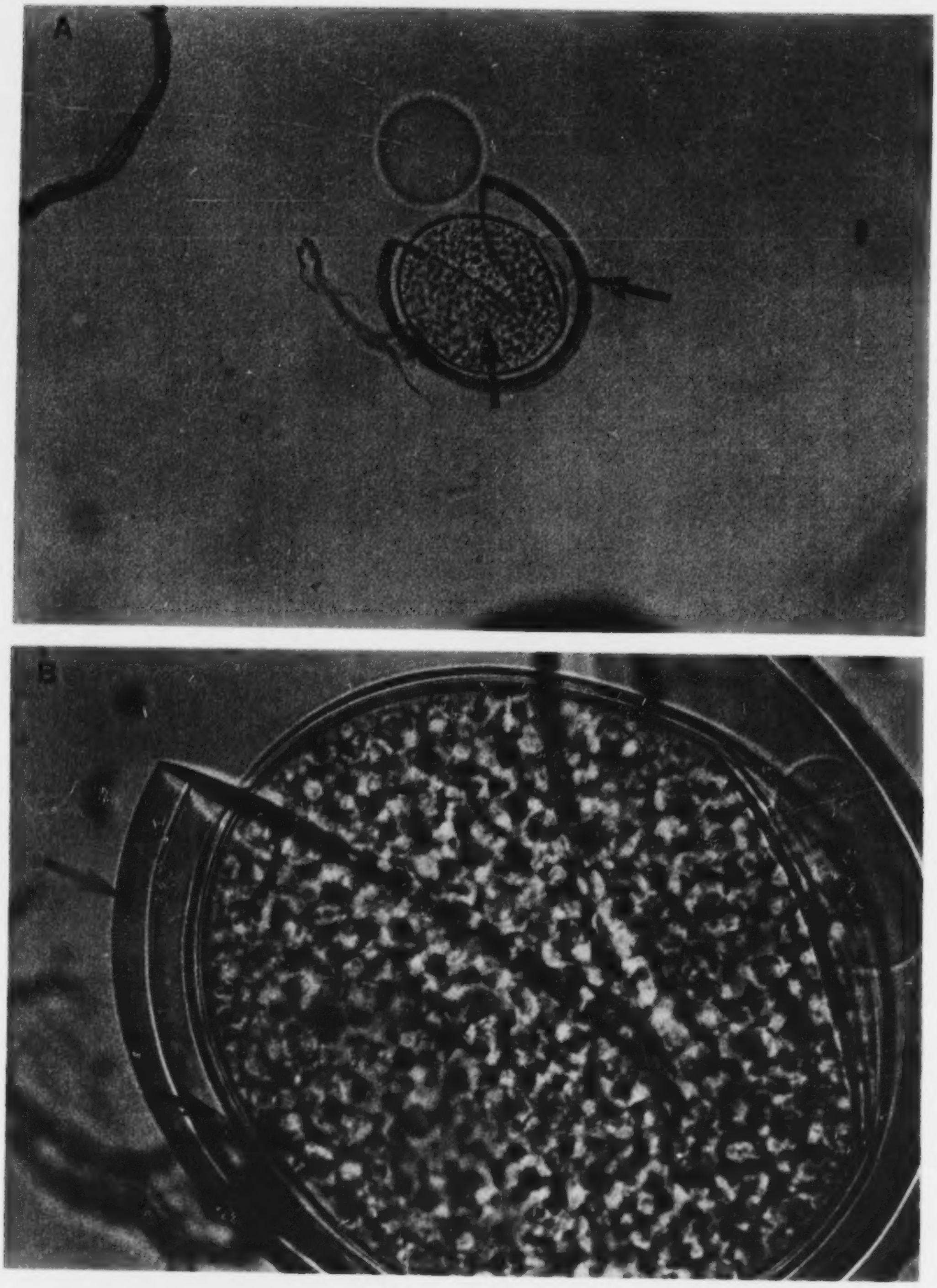
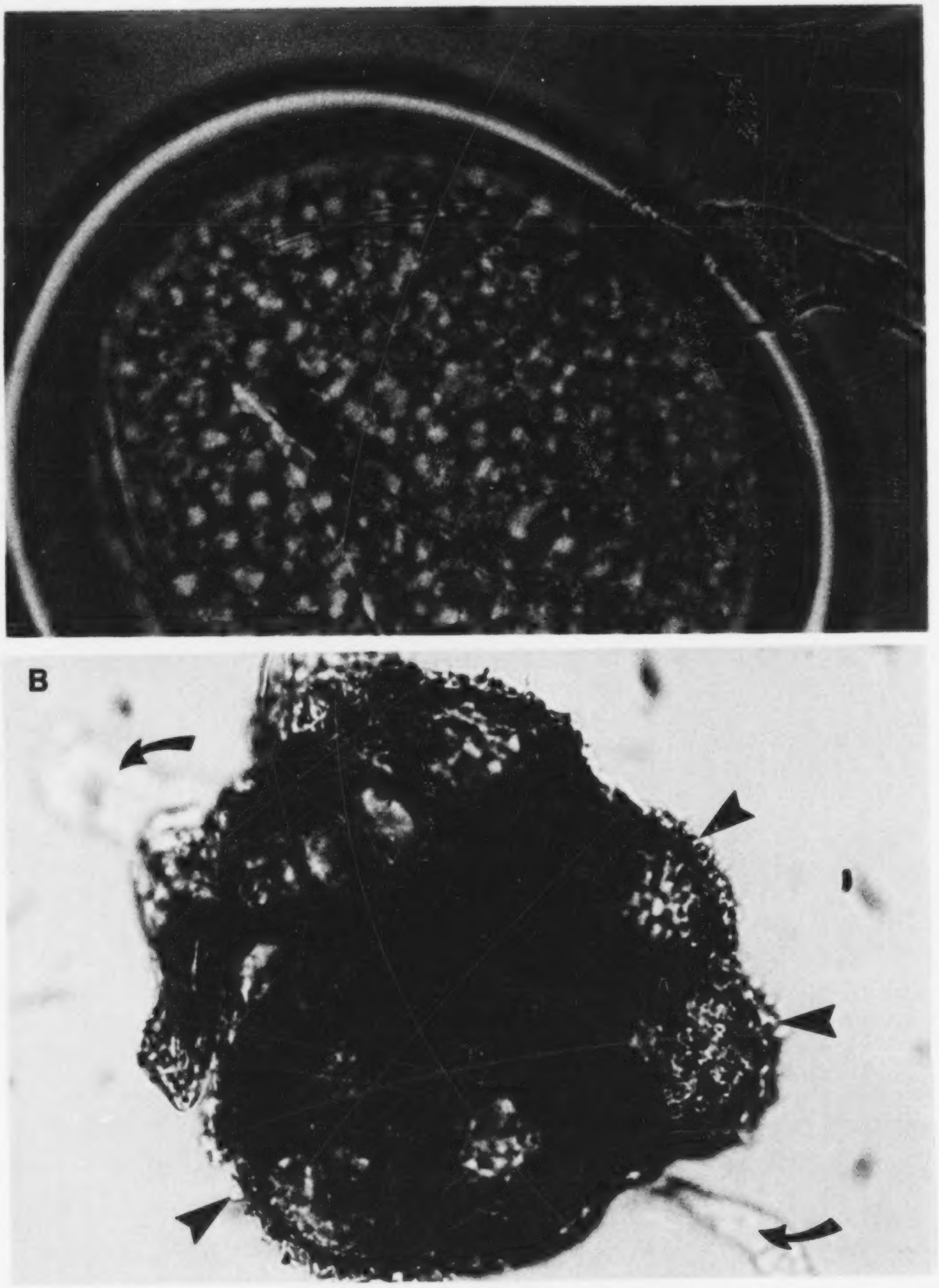

A
B

Fig. IV-20. Light micrograph of *Gigaspora margarita* spores isolated from soils from carnation growing areas of Kenya

A (x545) Note the bulbous hyphal attachment (arrowhead) which is septate below the suspensor-like cell (large arrowhead)

Note the projection into the spore from the suspensor-like cell

B (x200) Cluster of spiney vesicles presumed to be formed by this fungus. Note the spines (arrowheads) and hyphae (arrows)


B

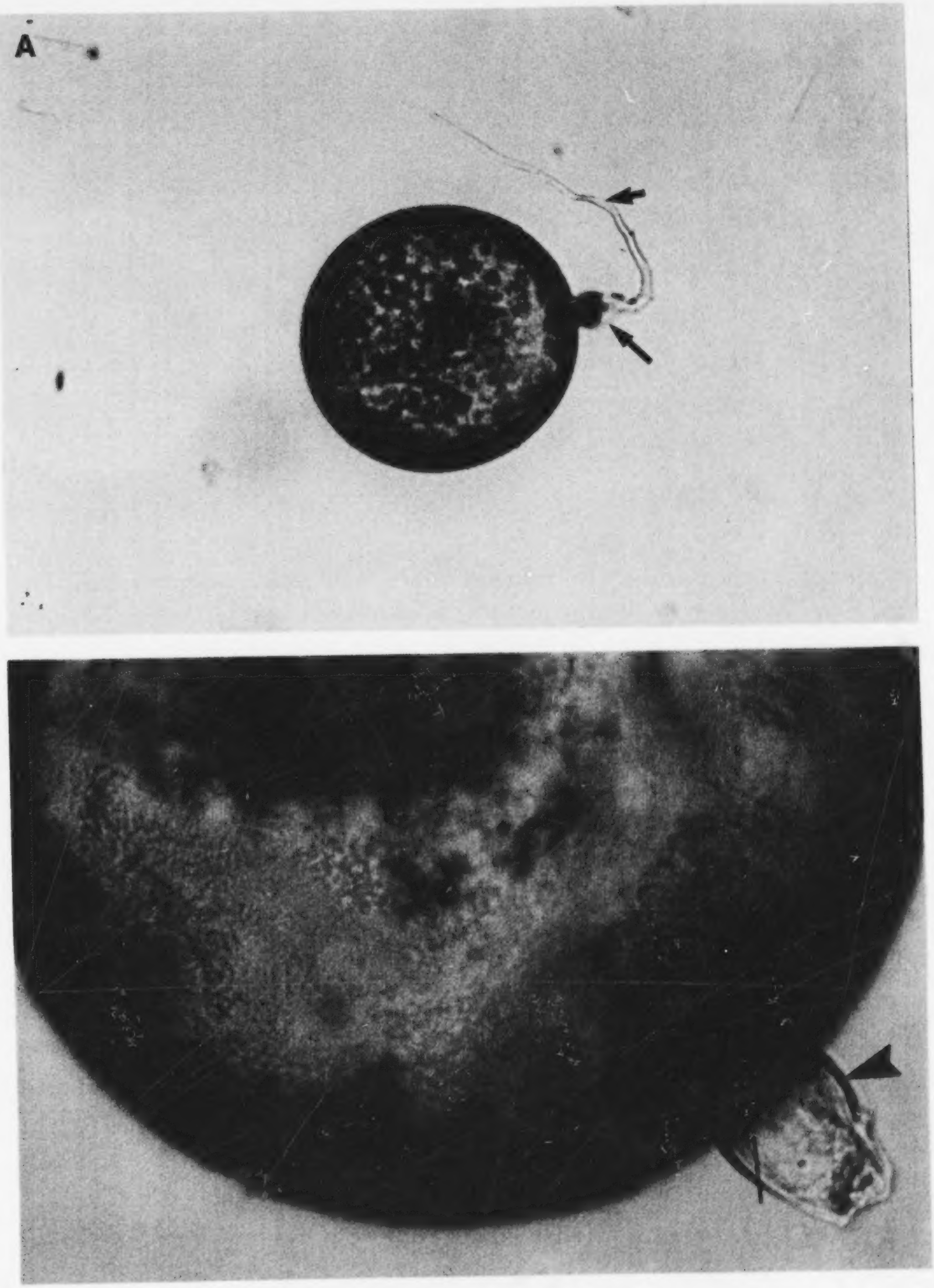

A

Fig IV-21. Light micrographs of *Gigaspora gigantea* spores isolated from soils from carnation growing areas of Kenya

A (x113) Note suspensor-like cell and subtending hypha

B Details of suspensor-like cell. Note the walls of the hypha (arrowheads) and the pore (arrow)

Fig IV-22. Light micrographs of *Gigaspora gigantea* spores isolated from soil samples from carnation growing areas of Kenya

A Details of suspensor-like cell (arrows)

B Details of wall structure. Note the laminate wall layers(arrow)

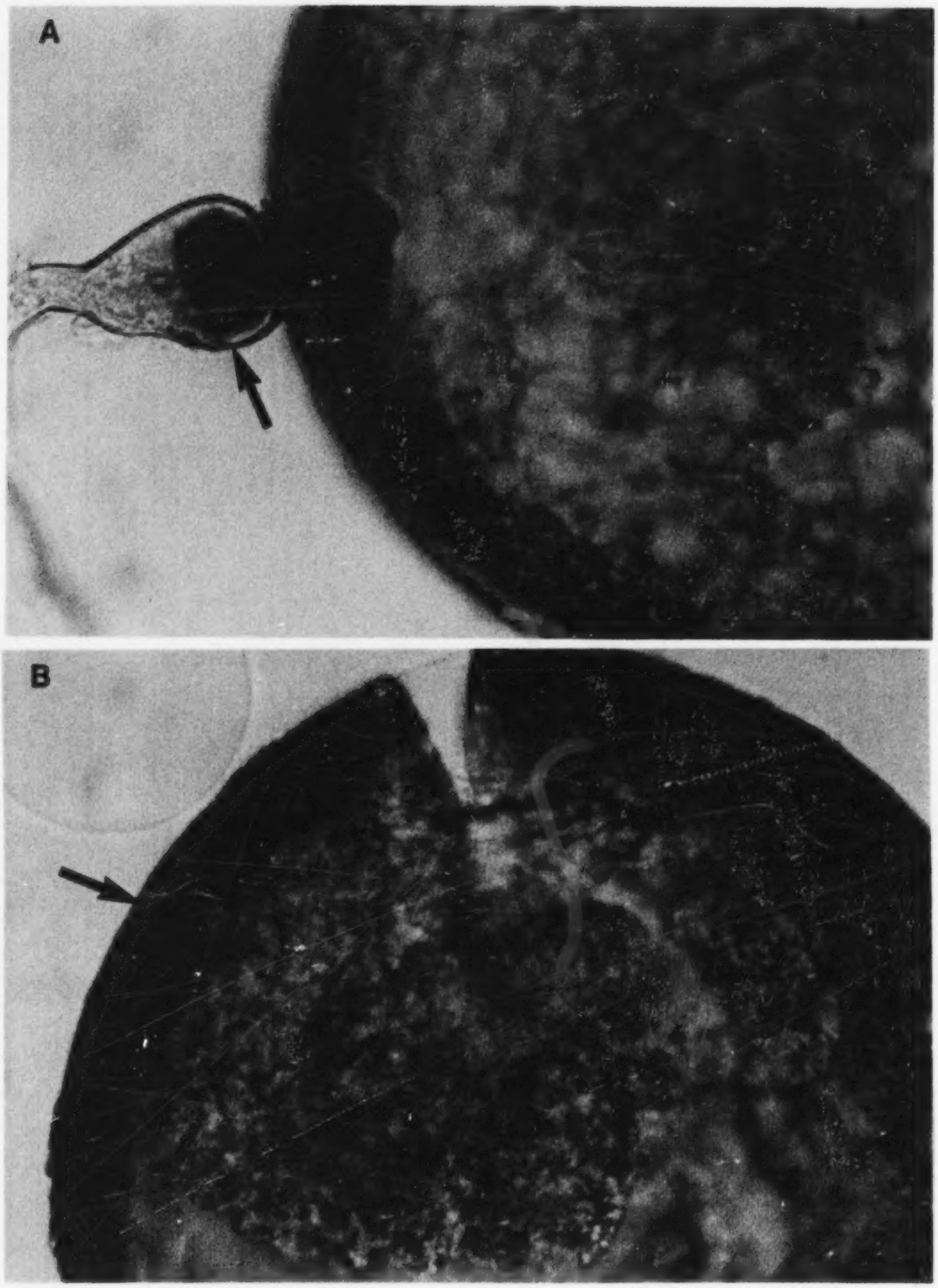
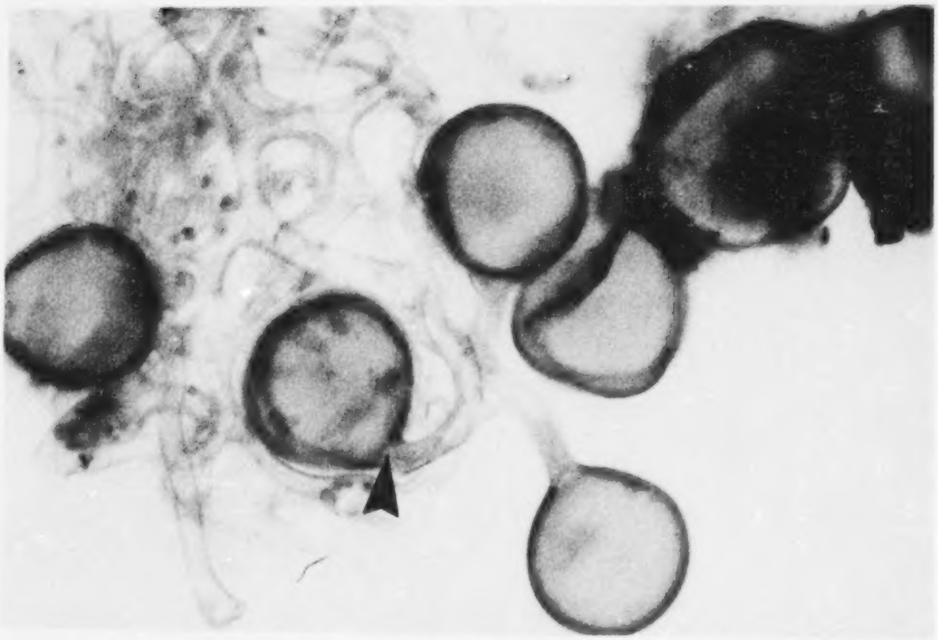

A
B

Fig. IV-23. (x400) Light micrographs of *Glomus mosseae* spores isolated from soils sampled from carnation growing areas of Kenya, showing loose clusters of spores
Note the flared subtending hyphae (arrowhead)



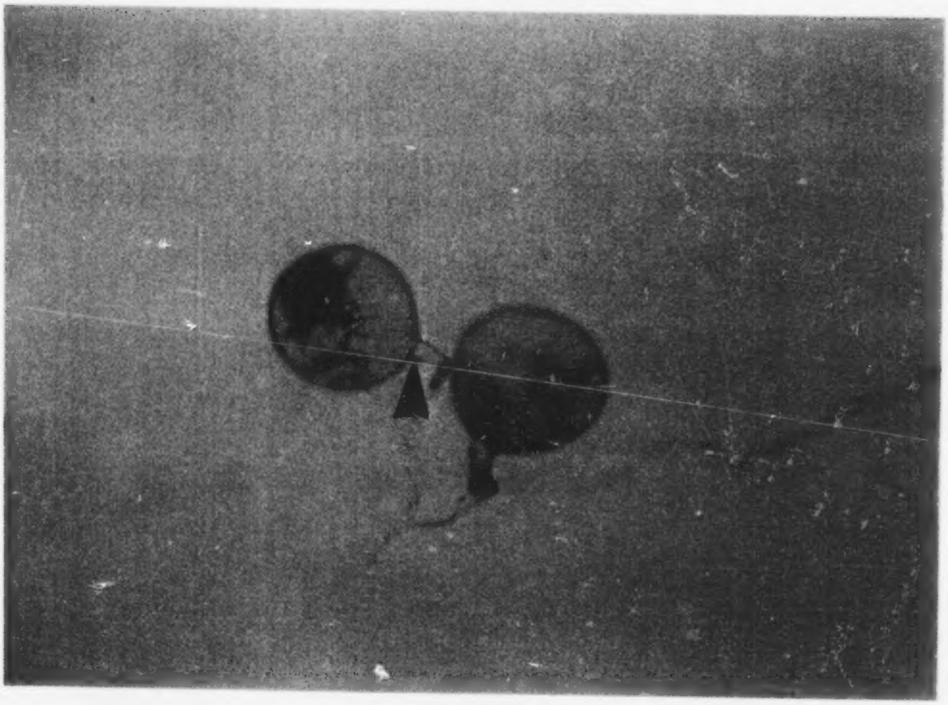


Fig. IV-24. Light micrographs of *Glomus mosseae* spores isolated from soils sampled from carnation growing areas of Kenya

A Details of spore wall

B (x550) Details of the funnel-shaped subtending hypha. Note the curved septum dividing it from the spore (arrow). Also note the collar (short arrow)

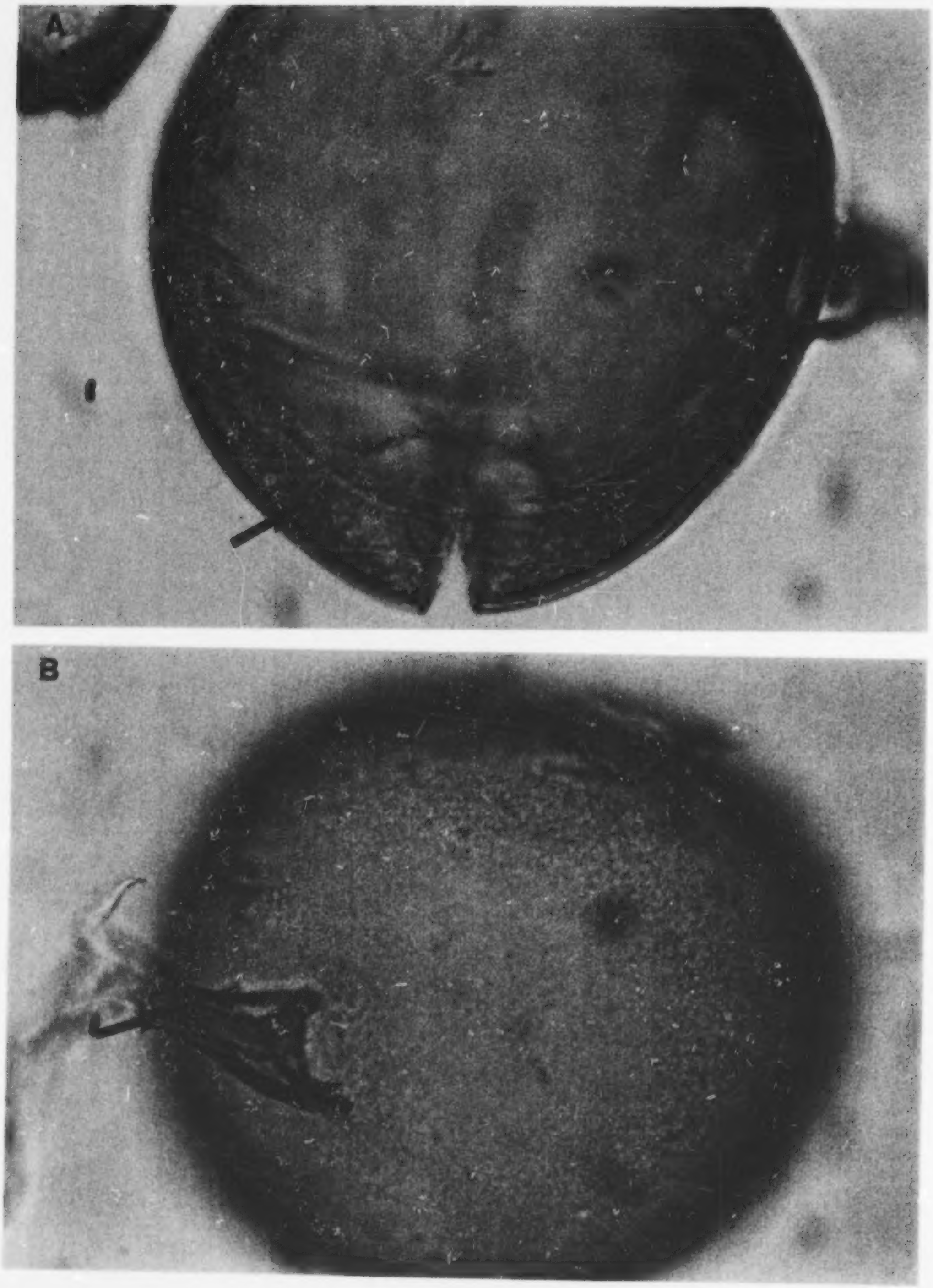
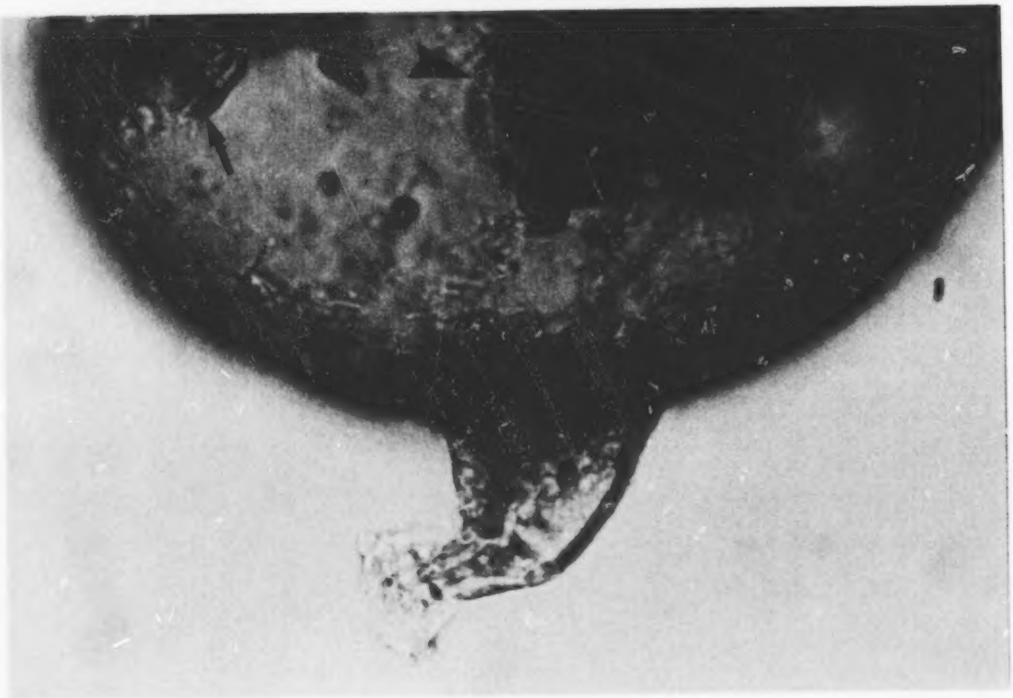

A
B

Fig. IV-25. Light micrographs of *Glomus mosseae* spores isolated from soils sampled from carnation growing areas of Kenya A Self-parasitized spore. Note the papillae (arrows) and the parasitizing spores (arrowheads)



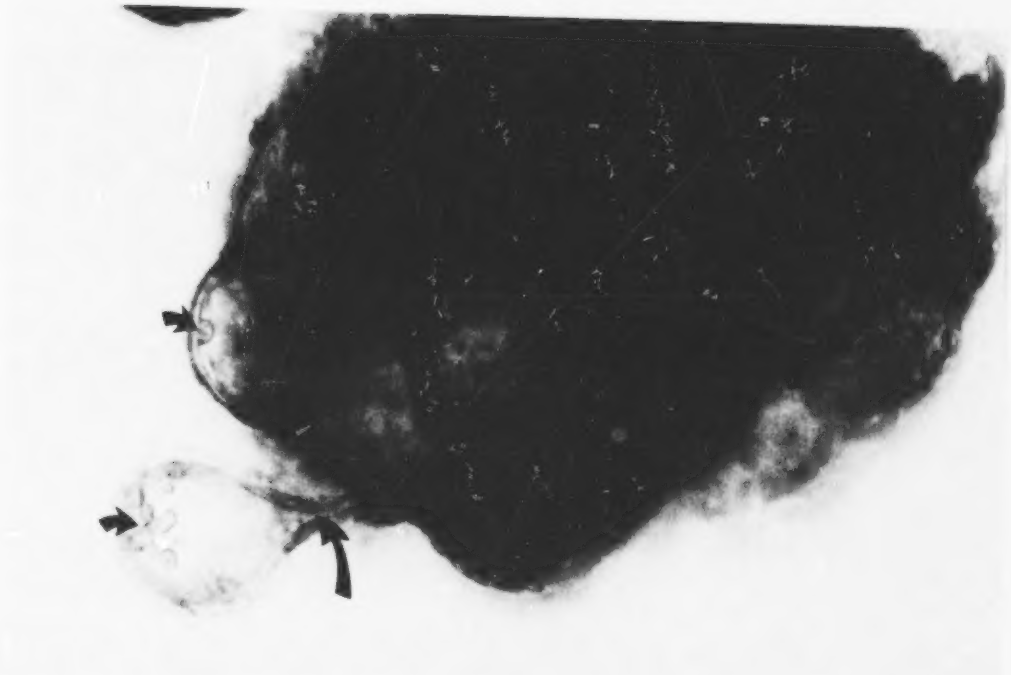


Fig IV-26. Light micrograph of a sporocarp of *Glomus rubiformis* (x400) isolated from soils sampled from carnation growing areas of Kenya

Note single spore showing open-pore hyphal attachment (arrow) and papillae (small arrows)

Fig. IV-27. Light micrographs of *Glomus tenebrosum*(?) isolated from soils sampled from carnation growing areas of Kenya

A Loose cluster of spores (x400) Note light-coloured subtending hyphae (arrowheads)

B (x370) Details of spore. Note the thick spore wall (arrows) and granular spore contents

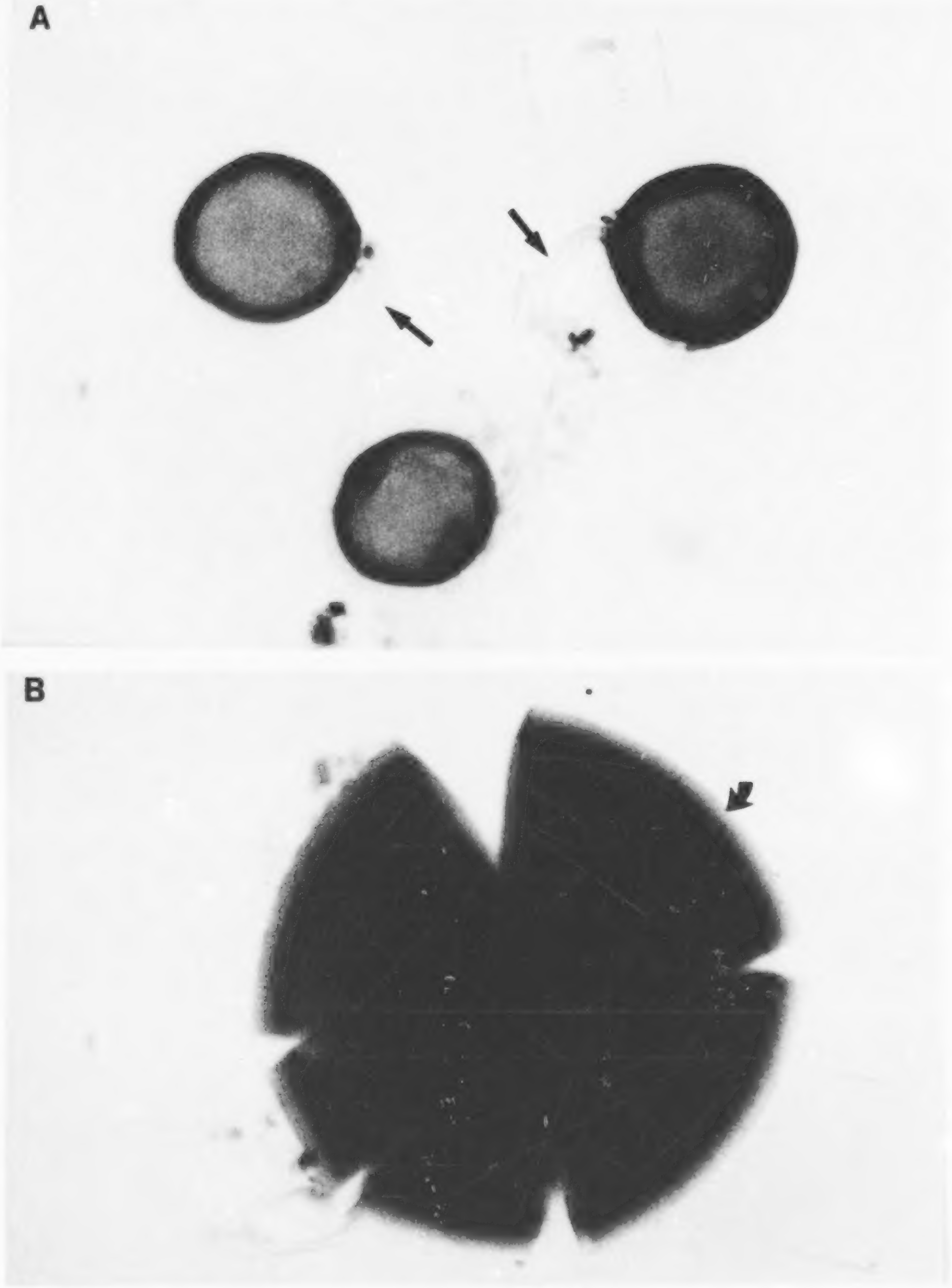

A
B

Fig. IV-28. Light micrographs of *Glomus tenebrosum*(?) isolated from soils sampled from carnation growing areas of Kenya (x350) Note parasitized spore in MPVLA

Fig. IV-29. Light micrographs of *Glomus tenebrosum*(?) isolated from soils sampled from carnation growing areas of Kenya

A Ellipsoid spores (x375). Note the presence of two wall layers (arrowheads)

B (350) Note the open pore (arrow)

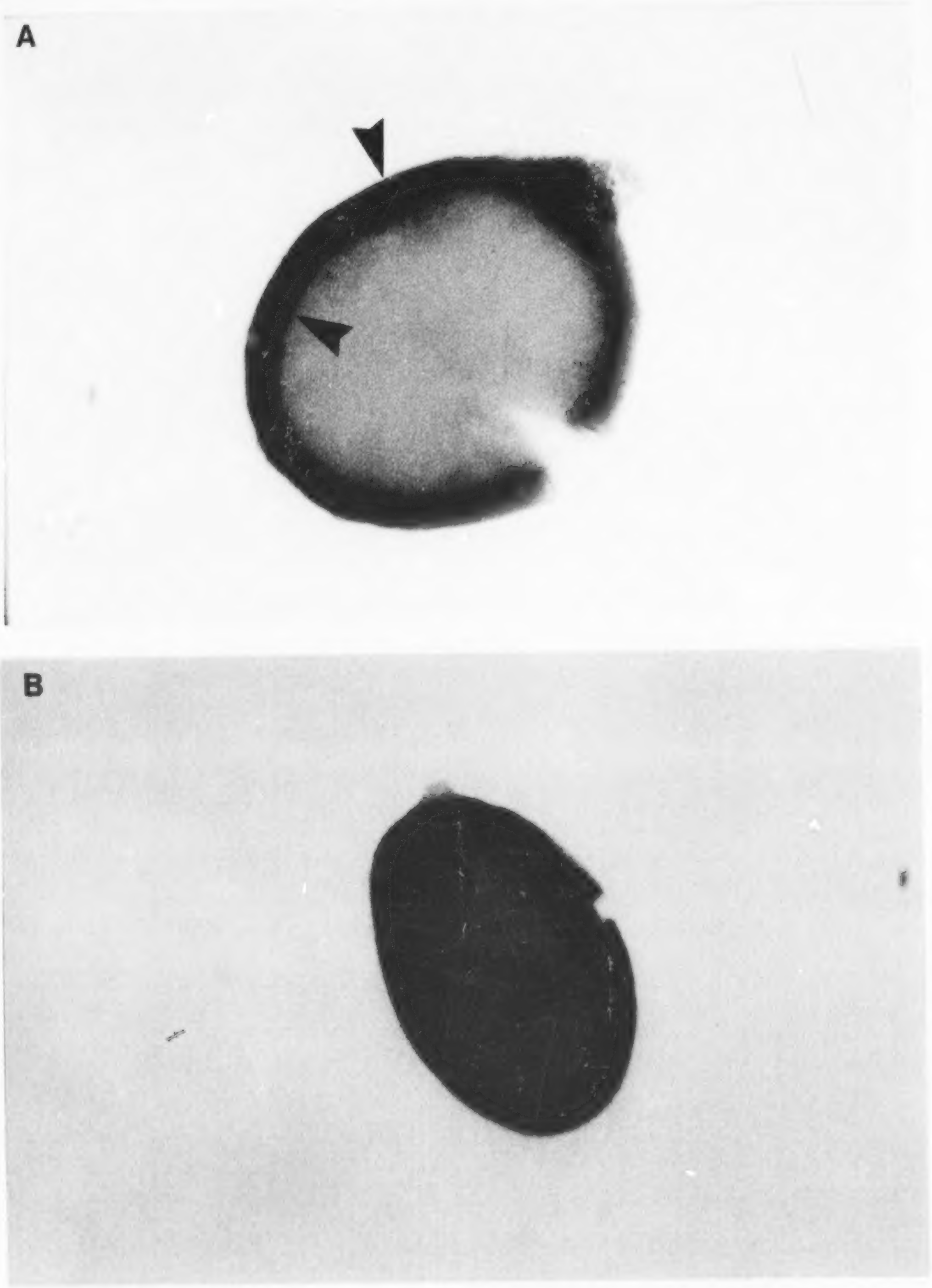

A
B

Fig IV-30. Light micrograph of *Glomus caledonium*(?) isolated from soils sampled from carnation growing areas of Kenya

A (x155) Note the branched subtending hypha (arrows) Note the evanescent outer wall which became red in Melzer's reagent (arrowhead)

B (x155) Note the loss of the outer evanescent wall

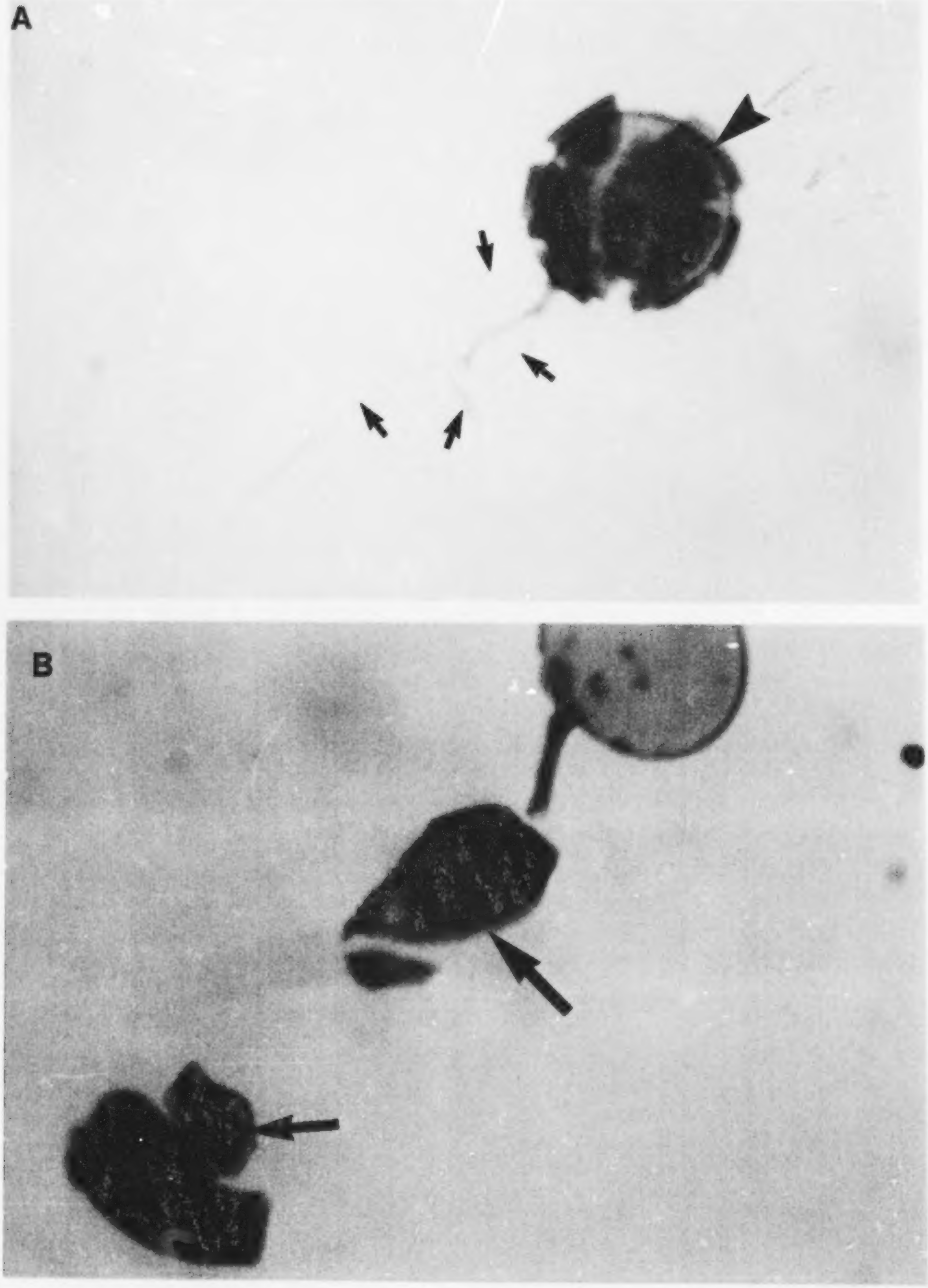

A
B

Fig IV-31. (x550) Details of fig. IV-30

Note the warty appearance of the inner wall (arrowheads)

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Chapter V

General Discussion and Conclusions

A. Cryopreservation of VAM Fungi and Taxonomy of VAM Fungi From Carnation Growing Areas in Kenya

The MTT vital stain failed to give typical reactions with *G. dimorphicum* and the *G. intraradices* spores, and did not differentiate between viable and non-viable spores of these two species. However, the vital stain successfully differentiated between viable and non-viable spores of *G. fasciculatum* and *G. mosseae*. An and Hendrix (1988) indicated that the MTT procedure gives a reliable estimate of viability for Endogonaceous spores isolated from field soils. The results of this study showed clearly that the MTT procedure should not be used indiscriminately to test all species of VAM fungi for spore viability. The efficacy of vital stains in viability tests differs among the stains for different plant species (Bulat, 1972), a phenomenon that may be carried over to fungal spores. Given the results of the present study,although the vital stain procedure does have great potential for quantitative determination of VAM spore viability, specific techniques may be needed for the different species. One should also keep in mind that VAM fungal spores have a high propensity for hyperparasitism, (Daniels and Menge, 1980) and that a parasitized dead VAM spore may therefore test viable. There is need for a reliable, repeatable method to quantitatively determine viability of VAM fungal spores of different species. The bioassay performed on revived VAM fungal spores that had been cryopreserved in liquid nitrogen, indicated that a large percentage of spores retained their viability, even after 13 months of cryopreservation. This study therefore showed that cryopreservation in liquid nitrogen may offer an alternative, space efficient and relatively easy method of preserving VAM fungal spores.

Fifteen different spore types were isolated from the Kenyan soils: *Acaulospora scrobiculata, Acaulospora denticulata, Entrophospora* sp., *Entrophospora infrequens, Glomus* sp., *Glomus lacteum, G. aggregatum, Glomus deserticola, G. intraradices, Gigaspora margarita, Gigaspora gigantea, Glomus mosseae, Glomus rubiformis, Glomus tenebrosum* and *Glomus caledonium*. Two of these *Acaulospora denticulata* and *Entrophospora* sp. were isolated from raw field soils collected from Naivasha, but not from greenhouse grown pot culture samples. This may have been because they did not multiply in culture, or if they did they were too few and were therefore not present in the samples observed. Failure to multiply may have been due to dormancy (Tommerup, 1983). Since these were mixed cultures, failure of the spores to multiply may also have been due to competition by other VAM fungi (Hepper et al. 1988), possibly for space and nutrients (Wilson and Tommerup, 1992). Infectivity of spores is known to differ both among (Thomson et al., 1990) and within species. Wilson (1984a) showed the differences in the infectivities of *Glomus fasciculatum*, *G. tenue*, and *Gigaspora decipiens* depended on inoculum density, implying intraspecific competition. Another explanation for failure of the spores to multiply could be that they needed association with certain micro-organisms which were absent in sterilized soils. Tylka et al. (1991) showed that three *Streptomyces* sp. stimulated germination of *Gigaspora margarita* and *Glomus mosseae* spores.

The rest of the spores in this study multiplied in pot cultures. However, some spores observed in Naivasha soil were not observed in Kiambu soils, while other spores were observed only in Kiambu soils. The fact that VAM fungi are obligate symbionts means that the distribution of VA mycorrhizas in cultivated soils are greatly affected by the species of crop grown (Hayman, 1987). Schenck and Kinloch (1980) showed that the crop species itself can exert a selective effect on which VAM species become predominant in a mixed indigenous population. The crops grown in the two carnation producing areas are slightly different, for example, bananas are grown in Kiambu, but not in Naivasha.

B. Etiology of vascular wilt of carnation in Kenya

Vascular wilt of carnation in Kenya was caused by *F. oxysporum* f. sp. *dianthi* and not *Phialophora cinerescens*. The latter pathogen was not isolated from the wilted carnations sampled. Although *Fusarium avenaceum* was isolated from the wilted carnations sampled, application of Koch's postulates established that this fungus did not cause vascular wilt of carnation, although it was pathogenic and is reported to cause Fusarium stem and bud rot (Nelson, 1960; Baker and Tammen, 1954). It was isolated from plants that also carried *F. o. dianthi* and the conclusion reached was that the two pathogens could infect the same plant concurrently. Some symptoms of Fusarium stem and bud rot could be confused with those of Fusarium wilt Baker et al. (1985) and this symptom similarity of the two diseases may have lead to an overestimation of the apparent vascular wilt disease severity in the fields surveyed.

Five F. oxysporum isolates differentiated on the basis of their gross morphology on half-strength PDA were isolated from the stems of wilted carnations and were arbitrarily named B, E, F, G, and H. When tested for pathogenicity, these isolates induced significantly different K_{max} disease severity levels under greenhouse conditions. Although these could all be isolates of one race of *F. o. dianthi*. (Demmink et al., 1989), the difference between K_{max} of Isolate B (92.5%) and Isolate F (20%), was great enough to suggest that the two sets of isolates have different virulence patterns. This could imply presence of more than one race of the pathogen (Armstrong and Armstrong, 1974). The amount of time that elapsed before onset of apparent symptoms was different for different isolates which was a further indication of a difference in virulence patterns of the five isolates. The differences in pathogenesis among the isolates also indicated the presence of more than one race (Baayen et al., 1988). In the absence of genetically pure differential hosts however, it was difficult to determine whether these isolates comprised different races, or if they comprised one variable race complex (Baayen et al., 1988).

C. Interaction of Fusarium Wilt of Carnation With VAM Fungi

The carnation cultivar Lolita was infected by *Glomus mosseae* and *G. intraradices*, but not by *G. dimorphicum*, indicating a cultivar by VAM spp. interaction effect for carnation. This effect was emphasized when a second experiment was carried out using three other carnation cultivars, Scania, Portrait, and Lavender Lace. None of these showed vesicle formation with either *G. mosseae* or *G. intraradices*. Lavender lace, however did show some vesicle formation when inoculated with *G. fasciculatum*. This disparity in response to VAM among host cultivars (genotypes) within the same species is not novel and has been reported. Mercy et al. (1990) described mycorrhizal colonization in cowpea to be host dependent and heritable. Results from this study indicated a high likelihood that infection of carnation by VAM, could be genotype dependent. This would indicate that it would be possible to improve carnation susceptibility through breeding. The problem here would be that selection for VAM susceptibility would have to compete with selection for floricultural characteristics. The cultivar Lolita formed both vesicles and arbuscules when inoculated with either *G. mosseae* or *G. intraradices*. Since arbuscules are considered to be the site of nutrient exchange with the host (Hirrel et al., 1978), this indicates that a functional symbiosis was established between plant and VAM fungus. Except for a few vesicles, arbuscules were not observed in the association between Lavender Lace and *G. fasciculatum*. In cultivars Scania and Portrait, points of mycorrhizal infection as well as aseptate hyphae strongly resembling that of VAM were observed, but no vesicles or arbuscules were observed. With the lack of arbuscule formation in these three cultivars, it is questionable whether a functional symbiosis was established.

Whatever level of association there was between the VAM fungi and the carnation cultivars, it did affect the slopes of disease progression curves of carnation Fusarium wilt. Infection with VAM enhanced disease severity in Lavender Lace indicating that susceptibility to VAM of Lavender Lace is not necessarily a beneficial trait. This phenomena of VAM fungi infection enhancing disease severity should caution one against indiscriminate use of VAM fungi in commercial situations without extensive field testing. For mixed cropping systems such as is the case with carnation culture in Kenya, it also emphasizes the need to study a given VAM fungus and its hosts, not only in isolation, but also in the ecosystem they are to function in. Inoculation of both Portrait and Scania with VAM fungi depressed disease levels despite extremely low VAM infection levels. This reduction of disease severity, although minor, may nevertheless be important from the point of view of integrated disease management. Systemic induced resistance in controlling Fusarium wilt in carnation has been described involving a *Pseudomonas* sp. isolate and a nonpathogenic *Fusarium* isolate. (Peer et al., 1991). Chitinolytic bacteria isolated from carnation rhizospheres and applied by dipping roots of carnation in cell suspension during transplanting, are also successful biocontrol agents against Fusarium wilt (Garibaldi and Gullino, 1987). Significant reduction in the number of diseased plants under field conditions by applying cell suspensions of

Arthrobacter sp. and *Serratia liquefaciens* has been reported (Sneh, 1981; Sneh et al., 1985). To date, use of chitinolytic bacteria has not been applicable in carnation culture at an industrial level. Deliberate contamination of VAM inoculum with the mentioned micro-organisms may aid in the application and establishment of these micro-organisms in carnation culture. Since the stock cultures are raised in sterilized soils, selected micro-organisms could be introduced and established before invasion and competition from aerial and soil contaminants becomes intense. One should take into consideration however, that some micro-organisms may inhibit germination of specific VAM fungi (Tommerup, 1985), and others stimulate it (Daniels et al., 1980). The micro-organisms must therefore be compatible with the VAM fungi used.

Parasitism of *G. intraradices* vesicles in Lolita roots was observed. This hyperparasitism is an important aspect in VAM research, as it could lead to variable results when different batches of the same mycorrhizal inoculum are used. In addition, roots of plants infected with *G. intraradices* were also infected with *Olpidium brassicae* and *Rhizoctonia* sp. These parasites could also have contributed to the low level of infection by *G. intraradices*.

Inoculation of carnation plants with VAM fungi did not improve their phosphorous nutrition. Observed improved growth of the carnation is therefore unlikely to have been due to improved phosphorous nutrition, but could be due to improved uptake of other nutrients. Uptake of nutrients such as K, Ca, Mg, Fe, Mn, Cu, Zn, Na, or B has been implicated in mycorrhizal assisted nutrition (Mosse, 1973). Production of growth promoting hormones by VAM fungi has also been implicated for improved plant growth of mycorrhizal plants (Krishna et al., 1981), although there has been no direct evidence of the transfer of these fungal hormones to the host plant in the symbiotic association.

Observations made from the SEM study were consistent with what has already been reported (Baayen, 1987; Harling et al., 1984; Baayen et al., 1989; Pennypacker and Nelson, 1972). In this study, occlussion plugs were found to contain calcium. The observed accumulations of calcium deposits that were mixed with gums could enhance the physical and chemical ability of the gums to block pathogen advancement. Calcium has been associated with induction of disease resistance in plants (Muchovej et al., 1980; Moore and Wills, 1967). In carnation, Blanc et al. (1983) reported that calcium deficiency at any stage of plant development increases Fusarium wilt. In the present study, it was observed that the resistant cultivar (Lavender Lace) showed more calcium deposition along cut surfaces of cell walls than did the other more susceptible cultivars. Baayen (1986) reported that hyperplasia in carnation is primarily a defence reaction associated with periderm formation and intended for localization of pathogens. Since calcium is a major cationic wall component of plant cells, this deposition could be indicative of actively dividing (hyperplastic) or enlarging (hypertrophy) cells, both of which indicate a resistance plant response (Baayen, 1986). Induction of disease resistance by calcium has also been attributed to the poor activity on calcium pectates by pathogens' pectinolytic enzymes. Several Fusarium wilt pathogens produce pectinases (Deese et al., 1962; Edington et al., 1961; Heitefuss et al., 1960) which attack the cell walls and middle lamellae. This type of of cell wall degeneration was noticed in this study, more so in the susceptible Scania and Portrait cultivars than in the more resistant Lavender Lace. This study therefore supported the finding by Blanc et al (1983) that calcium may reduce disease severity.

D. Conclusions

1. Carnation wilt in Kenyan fields is caused by a mixture of both *F. o. dianthi* and *F. avenaceum.*
2. It is possible that there is more than one races of *F. o. dianthi* infecting the carnation and one of them could possibly be race 2.
3. The MTT vital stain can be used to differentiate viable from non-viable spores of some species of endogonaceous fungi but not others.
4. Cryopreservation in liquid nitrogen may offer an alternative method of endogonaceous spore storage.
5. The carnation growing areas of Kenya have a wide variety of vesicular-arbuscular mycorrhizal fungi. Fifteen different species spanning the genera *Glomus, Acaulospora, Entrophospora,* and *Gigaspora* were isolated from the soils.
6. The cultivar Lolita developed a functional symbiosis with *G. intraradices* and *G. mosseae*, but not with *G. dimorphicum.*
7. Inoculation with VAM of Lavender lace increased severity of Fusarium wilt in this cultivar, while in Portrait and Scania, the disease severity was depressed by VAM infection.

8. Inoculation of carnation with VAM fungi did not result in any significant difference in the dry weights and the phosphorous content of the infected plant shoots.

E. Future Work

1. Further studies on the race identification using genetically pure differential hosts or restriction fragment length polymorphisms (RFLP) need to be carried out to determine the *F. o. dianthi* races present in Kenyan carnation culture.
2. Quantitative studies of the efficacy of cryopreservation of VAM spores in liquid nitrogen are also required.
3. Studies on the effect of some of the VAM species indigenous to the Kenyan carnation growing areas on Fusarium wilt of carnation will help identify VAM fungi adapted to the local ecological conditions.
4. A systematic survey and identification of the VAM fungi species present in the Kenyan carnation growing region need to done before studies on the interaction of VAM fungi with carnation can be done.

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